MOLECULAR CHARACTERIZATION OF CITRUS TRISTEZA VIRUS GENES AND THEIR USE IN PLANT TRANSFORMATION

By

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

ABSTRACT vi

CHAPTER

1. INTRODUCTION 1

2. DETECTION OF THE *IN VIVO* EXPRESSION OF THE P27, P20 AND P18 PROTEINS 8

Introduction 8
Materials and Methods 9
Materials 9
Plant Material 10
Cloning of CTV ORFs into pUC118 10
Subcloning of CTV ORFs in the Expression Vector 16
DNA Sequencing 17
Bacterial Expression of CTV Proteins . . . 18
Production of Polyclonal Antisera 19
Detection of CTV Proteins in Infected Citrus Tissue 20
Results 22
Cloning of CTV Genes 22
Expression of Proteins 23
Detection of p27 24
Detection of p20 31
Detection of p18 31
Discussion 33

3. SEQUENCE ANALYSIS OF THE P27 ORF 40

Introduction 40
Materials and Methods 41
Materials 41
Virus Isolates 42
Cloning and Sequencing 42
Single Stranded Conformation Polymorphism (SSCP) 44

Results 44
Sequencing 44
Single Stranded Conformation Polymorphism 54
Discussion 63

4. TRANSFORMATION OF SWEET ORANGE WITH P27 AND P20 67

Introduction 67
Materials and Methods 70
Materials 70
Cloning of p27 Into the Transformation Vector 70
Cloning of p20 Into the Transformation Vector 71
Transformation of pVF plasmids into *Agrobacterium* 73
Screening of Transformed Bacteria 73
Transformation, Selection and Regeneration of Citrus Plants 75
Results 80
Cloning of CTV Genes Into the Transformation Vectors 80
Transformation and Regeneration of Sweet Orange 80
Discussion 87

5. SUMMARY AND CONCLUSIONS 93

LITERATURE CITED 95

BIOGRAPHICAL SKETCH 105

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Nucleotide sequence analysis of citrus tristeza closterovirus (CTV) revealed the presence of 12 possible open reading frames (ORFs). The ORF immediately upstream of the coat protein (CP) gene encodes a protein of calculated MW of 27.4 kDa (p27). The deduced amino acid sequence indicated that this gene product is homologous to the CP (41% similarity). Two other genes located toward the 3' end of the CTV genome encode proteins of 18.3 (p18) and 20.5 (p20) kDa, respectively. Deduced amino acid sequence comparisons with protein databases did not reveal any significant relationship that might indicate the possible function of these proteins. The objectives of this research were: 1) to determine if p27, p20 and p18 proteins were expressed in CTV-infected citrus tissue, 2) to identify the sequence variability of the p27 ORF among CTV strains with different biological properties and to

determine any possible differences that could correlate with virulence, and 3) to transform citrus plants with p27 and p20 genes in an attempt to confer resistance to CTV. Fusion proteins for p27, p20 and p18 were produced in *Escherichia coli* and used to raise polyclonal antibodies. Western blot analyses using the antisera indicated that p27 and p20 are expressed in CTV-infected citrus, but not in uninfected plants. Tissue fractionation studies revealed that p27 accumulates in cell wall enriched fractions, whereas p20 accumulates in the soluble protein fraction. The expression of p18 was not detected in CTV-infected citrus tissue. The p27 protein also was detected using tissue blot analysis. RNA sequence homologies of the p27 gene among CTV isolates were between 86 and 99%. The deduced amino acid sequence homologies were 93% or higher. Phylogenetic analysis showed mild and severe, quick decline isolates grouping separately. *Agrobacterium*-mediated plant transformation experiments were performed using p27 and p20 genes. Approximately 6% of the adventitious shoots obtained on selection media with kanamycin were GUS positive. Between 31 and 37% of the GUS positive shoots were positive using PCR analysis with primers specific to p27 or p20, suggesting that some plants are transformed with each of the genes.

CHAPTER 1
INTRODUCTION

Citrus tristeza was initially recognized as a decline disease of citrus scions propagated on sour orange (*Citrus aurantium* L.) rootstock (Lee and Rocha-Peña, 1992). This disease, caused by citrus tristeza closterovirus (CTV), is one of the major diseases affecting citrus, and an excellent example of an agricultural problem created by man (Bar-Joseph et al., 1989). CTV is now known to cause a variety of symptoms depending on the virus strain and host. Perhaps the most economically important and obvious CTV symptom is the decline of sweet orange [*C. sinensis* (L.) Osbeck], grapefruit (*C. paradisi* Macf.) and mandarin (*C. reticulata* Blanco) grafted on sour orange rootstock (Garnsey et al., 1987; Bar-Joseph and Lee, 1990). Stem pitting in limes [*C. aurantifolia* (Christm.) Swingle], grapefruit and some sweet oranges is another important reaction and a limiting factor to production in parts of Brazil, South Africa and Australia (Garnsey et al., 1987; Bar-Joseph and Lee, 1990; Lee and Rocha-Peña, 1992). In addition, some CTV strains can induce seedling yellows when inoculated to sour orange, grapefruit and lemon (*C. limon* Burm.f) seedlings. The importance of this symptom is not known, except for CTV strain differentiation, because it

normally is not observed in the field (Garnsey et al., 1987; Bar-Joseph and Lee, 1990). Some mild strains, on the other hand, do not induce any noticeable symptoms in most of the cultivated varieties (Garnsey et al., 1987; Bar-Joseph and Lee, 1990). Several strains can be present in the same tree, as has been determined by cross protection experiments (Powell et al., 1992) and double-stranded RNA analysis (Moreno et al., 1991). This makes the use of biological methods, such as cross-protection, more difficult to control the disease, although they are still possible (Costa and Muller, 1980).

CTV is present in most areas of the world where citrus is grown. In South America it was first described in the 1930s. Less than two decades later CTV caused the destruction of most of the citrus industries in Argentina, Brazil and Uruguay, killing about 25 million trees planted on the susceptible sour orange rootstock (Muller and Costa, 1992). The spread of the disease and its impact is increased by the presence of its most efficient vector *Toxoptera citricida* (Kirkaldy), the brown citrus aphid. A well documented case occurred in Venezuela. The disease was reportedly present in the country in the mid-'50s without having a major impact. In 1976 *T. citricida* was first found in Venezuela in regions bordering with Colombia and Brazil (Geraud, 1976). Two years later it was widespread throughout the country. In 1980 the first outbreak of CTV was reported, and since then, at least 6

million citrus trees on sour orange rootstock have died from tristeza (Mendt, 1992).

In Florida, approximately 20-25 million trees of sweet orange, grapefruit and mandarin are planted on the susceptible sour orange rootstock (Garnsey, 1991). CTV has been present in Florida since the '50s, and both mild and decline inducing strains have been reported (Brlansky et al., 1986). In addition, 8-10 million trees of grapefruit are also susceptible to stem pitting strains, which have not been detected yet in Florida (Garnsey, 1991). The aphid *T. citricida* is still not present, although other less efficient vectors such as *Aphis gossypii* Glover and *A. citricola* van der Goot are widespread in Florida. However, *T. citricida* has continued its northward movement from South America. In recent surveys in Central America and the Caribbean the aphid was reported in Nicaragua (Lastra et al., 1991) and Cuba (Yokomi et al., 1994). *T. citricida* thus represents a serious threat to the citrus industries of Florida and the United States, as well as Mexico, Cuba and other Caribbean countries. This is because of its efficiency to vector CTV (6 to 25 times more efficient than *A. gossypii*; Yokomi et al., 1994), especially with the decline and stem pitting strains which are not efficiently vectored by the aphid species present in Florida (Yokomi, 1990).

Transmission of CTV by aphids is in a semipersistent manner (Bar-Joseph and Lee, 1990). It also is transmitted by

budding and grafting (Bar-Joseph and Lee, 1990). Mechanical transmission is difficult and only possible when concentrated preparations are used and slash inoculated into the stems of young citrus plants (Garnsey et al., 1977).

CTV is a phloem-limited virus. Inclusion bodies are observed inside the phloem, phloem fiber and parenchyma cells adjacent to sieve tubes (Schneider, 1959; Brlansky et al., 1988). They also are detected by immunofluorescence using antibodies to CTV coat protein (CP) (Brlansky et al., 1988), indicating that the inclusion bodies contain virus particles. These virus particles are flexuous, 1900-2000 nm long and 10-11 nm in diameter (Bar-Joseph and Lee, 1990).

The genome of CTV is a single-stranded, positive sense RNA 19,296 nucleotides in length (Bar-Joseph and Lee, 1990; Karasev et al., 1995). Sequencing of the entire genome of the Florida, severe, quick decline strain T36 indicates the presence of 12 possible open reading frames (ORFs) (Fig. 1) coding for 17 proteins (Pappu et al., 1994; Karasev et al., 1995). In the 5′ to 3′ direction the first ORF potentially codes for a polyprotein with an estimated MW of 349 kDa that encodes the putative domains for two papain-like proteases, a methyltransferase and a helicase (Karasev et al., 1995). This polyprotein is speculated to undergo autoproteolytic cleavage to produce three fragments of 54, 55 and 240 kDa, respectively (Karasev et al., 1995). The second ORF encodes a putative RNA-dependent RNA polymerase of a calculated MW of 57 kDa that is

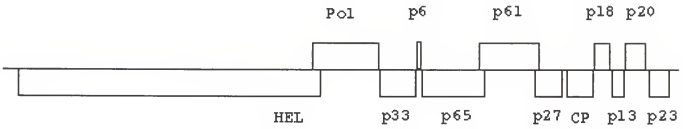


Figure 1. Genomic organization of the citrus tristeza virus genome according to Pappu et al. (1994) and Karasev et al. (1995). The ORFs are represented as rectangles and the putative gene products are indicated. (HEL, helicase; Pol, polymerase; CP, coat protein).

proposed to be expressed by ribosomal frameshift of the first ORF, resulting in a polyprotein of 401 kDa (Karasev et al., 1995). This polyprotein also would undergo autoproteolytic cleavage producing a 292 kDa fragment with domains for methyltransferase, helicase and RNA polymerase (Karasev et al., 1995). The third ORF potentially encodes a 33 kDa (p33) protein (Karasev et al., 1995) of unknown function, followed by a small ORF encoding a 6.4 kDa protein with a highly hydrophobic domain (Dolja et al., 1994). ORFs 5 and 6 code for proteins of 64.7 (p65) and 61.1 (p61) kDa, respectively (Pappu et al., 1994). Sequence comparisons indicate conservation of several motifs between p65 and the hsp70 group of heat shock proteins (Pappu et al., 1994). Similarly, p61 contains a C-proximal domain that is present in another group of heat shock proteins, the hsp90 group (Pappu et al., 1994). These two proteins have been detected in CTV-infected citrus tissue (S.S. Pappu, personal communication).

The following ORFs, 7 and 8, encode proteins of calculated MW of 27.4 (p27) and 24.9 kDa, respectively (Pappu et al., 1994). ORF 8 has been identified as the CP gene (Sekiya et al., 1991; Pappu et al., 1993b). Based on their deduced amino acid sequence, p27 shows 41% similarity with the CP (Pappu et al., 1994). ORFs 9 to 12 potentially encode proteins with calculated MW of 18.3 (p18), 13.2 (p13), 20.5 (p20) and 23.7 (p23) kDa, respectively (Pappu et al., 1994). The functions of these proteins are not known, and deduced

amino acid sequence comparisons with protein databases do not reveal any significant relationships (Pappu et al., 1994). Only p23 seems to contain a motif similar to some RNA-binding proteins (Dolja et al., 1994). Fig. 1 indicates the genome organization of the 12 potential ORFs described above.

The identification of the functional ORFs of CTV will facilitate understanding the mechanisms of gene expression, replication and pathogenesis of this important virus, and eventually enable the development of better disease control strategies. The expression of viral genes in transgenic plants, for example, has been a useful strategy to produce plants resistant to several diverse viruses (Powell Abel et al., 1986; Beachy et al., 1990; Pappu et al., 1995).

In this research the expression of the CTV genome was studied to increase the information available on this virus. The specific objectives of this research were as follows:

1) To determine if the p27, p20 and p18 proteins were expressed in CTV-infected citrus tissue.
2) To identify the sequence variability of the p27 ORF among CTV strains with different biological properties and determine any possible differences that could correlate with virulence.
3) To transform citrus plants with the p27 and p20 genes in an attempt to genetically engineer CTV resistance in citrus.

CHAPTER 2
DETECTION OF THE *IN VIVO* EXPRESSION OF THE P27, P20 AND P18 PROTEINS

Introduction

There is significant resemblance between the genomic organization of CTV and two other members of the closterovirus group, beet yellows virus (BYV) and the bipartite lettuce infectious yellows virus (LIYV). CTV ORFs 1 (helicase), 2 (polymerase), 4 (small hydrophobic protein), 5 (p65-hsp70), 6 (p61-hsp90), 7 (p27-CP homologue) and 8 (CP) have homologous ORFs in BYV and LIYV in similar positions (Dolja et al., 1994; Klaassen et al., 1995; Pappu et al., 1994). Interestingly for LIYV, the CP homologue is located downstream of the CP (Dolja et al., 1994; Klaassen et al., 1995). Another CTV ORF, p20, shows similarity in the deduced amino acid sequence only to p21, the 3' terminal ORF in BYV (Pappu et al., 1994). The CTV ORFs 3 (p33), 9 (p18) and 12 (p23) do not have homologues in the two other closteroviruses.

The fact that some of the CTV ORFs have homologues in BYV and LIYV is a strong indication, but does not demonstrate, that those ORFs are functional *in vivo*. The detection of subgenomic RNAs for 9 of the 12 ORFs, including p27, p20 and p18 (Hilf et al., 1995) also supports that they are expressed

in vivo. The final evidence, however, is the detection of the protein products in infected tissue.

In order to investigate whether p27, p20 and p18 are expressed in CTV infected plants the genes were separately cloned into *Escherichia coli* expression vectors. The expressed proteins were used to raise polyclonal antibodies to probe for the proteins in CTV-infected citrus tissue.

Materials and Methods

Materials

Reagents were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO). Enzymes and lambda DNA marker were purchased from Promega Corporation (Madison, WI). Some restriction enzymes, agarose and low melting point (LMP) agarose were from GIBCO BRL (Gaithersburg, MD). Phenol (equilibrated with 0.1 M Tris-HCl, pH>7.8), T4 polynucleotide kinase, dNTPs and Sequenase Version 2.0 DNA sequencing kit were from Amersham (Arlington Heights, IL). The nucleotide [α-^{35}S]-dATP and the Renaissance chemiluminescent detection kit for Western blots were from DuPont NEN Research Products (Boston, MA). Protein standards SDS-6 and SDS-7 were from Sigma Chemical Company. Electrophoresis apparatus for small gels (7 x 8 cm), Mini-PROTEAN II dual slab cell, was from Bio-Rad (Richmond, CA). Electrophoretic transfer unit, Mini Trans-Blot, was also from Bio-Rad. The larger electrophoresis unit (14 x 14 cm), VAGE,

was from Stratagene (La Jolla, CA). The pETH-3b expression vector was kindly provided by D.R. McCarty. The expression clone for the CP was kindly provided by M.L. Keremane.

Plant Material

Mexican lime [*C. aurantifolia* (Christm.) Swingle] plants infected with CTV T36 were maintained in greenhouses at the University of Florida (Gainesville, FL).

Samples of citrus infected with citrus ringspot virus, citrus variegation virus, citrus leaf rugose virus, psorosis A and concave gum were kindly provided by R.F. Lee.

Cloning of CTV ORFs into pUC118

Extraction of viral RNA templates. Total nucleic acids were extracted from citrus leaves infected with the Florida severe, quick decline strain T36, using the procedure described by Pappu et al. (1993c). Approximately 1 cm^2 of tissue was ground in a microcentrifuge tube with a sterile blunt stick (2 x 147 mm) in liquid nitrogen. The samples were thawed in 0.3 ml of extraction buffer [0.1 M Tris-HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% sodium dodecyl sulfate (SDS)]. An equal volume (0.3 ml) of a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) were added, followed by vortexing for a few seconds. The samples were incubated at 70°C for 5 min and then centrifuged at 14,000 rpm for 5 min at room temperature. The aqueous upper phase was saved.

The template was further purified using Sephadex G-50 spin-column chromatography, according to the procedure described in Sambrook et al. (1983). The column (a 1 ml syringe barrel), containing hydrated, sterile Sephadex, was equilibrated by washing three times with 100 µl of sterile TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 100 µl of extract applied to it. The column was centrifuged at 1,600g for 4 min at room temperature, and the effluent collected in a microcentrifuge tube. The effluent was used immediately for reverse transcription coupled with polymerase chain reaction (RT-PCR) amplification or quickly frozen in liquid nitrogen and stored at -80°C for later use.

PCR amplification. The CTV genes were amplified using RT-PCR (Saiki et al., 1988) and specific primers for each gene. Table 1 shows the primers, derived from the T36 sequence (Pappu et al., 1994), used to amplify each of the genes. The RT-PCR reactions were performed successively in a single microcentrifuge tube in a total volume of 100 µl with the following components: 10 mM Tris-HCl pH 9.0 (at 25°C), 50 mM KCl, 0.1% Triton X-100, 10 mM dithiothreitol (DTT), 2.5 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, RNasin (40 u), AMV reverse transcriptase (19 u), Taq polymerase (2.5 u) and primers at a final concentration each of 100 pM. Normally, 30 to 50 µl of the purified template were used in each reaction. It was previously denaturated at 70°C for 3 min and quick chilled on ice for a few min. The RT-PCR

Table 1. Description of the primers used for cloning CTV ORFs p27, p20 and p18.

ORF	PRIMER[a]	Tm[b] (°C)
p27	5′ **AAGCTTCTAGA**ACCATGGCAGGTTATACAGTAC 3′	54
	5′ CTATAAGTACTTACCCAAATC 3′	56
p20	5′ **AAGCTTCTAGA**ACCATGCGAGCTTACTTTAGTG 3′	54
	5′ CTACACGCAAGATGGAGA 3′	58
p18	5′ **AAGCTTCTAGA**ACCATGTCAGGCAGCTTGGG 3′	54
	5′ CTAAGTCACGCTAAACAAAG 3′	56

a Upper sequence is the genome sense primer. Lower sequence is the genome antisense primer. Bold letters are HindIII (AAGCTT) and XbaI (TCTAGA) restriction sites. The start codon is underlined.
b Tm= calculated melting temperature.

mixtures with the template RNA were incubated in an automatic thermocycler at 42°C for 45 min, followed by 40 cycles of incubations at 94°C for 1 min, 45°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 10 min. The same parameters were used for the amplification of all three ORFs.

After the PCR reaction was completed, a 5 µl aliquot was electrophoresed in a 0.8% agarose gel in TBE buffer (9 mM Tris-borate, 2 mM EDTA) to determine the amplification of the DNA fragment of the correct size. Lambda DNA digested with HindIII was used as a molecular weight marker.

Purification of the PCR products. Once the fragments of interest were detected, the rest of the PCR samples (95 µl) were electrophoresed in 0.8% LMP agarose in TBE buffer at 4°C and 50 to 75 V for about 1 h. The DNA bands of interest were excised from the gel with a sterile blade under UV light and transferred to a microcentrifuge tube. TE buffer was added to a final volume of 0.6 ml. Samples were then incubated at 65°C for 7 min to melt the agarose, and then extracted with phenol, phenol: chloroform, and chloroform followed by ethanol precipitation according to Sambrook at al. (1989). The final DNA pellet was resuspended in 10 µl of sterile distilled water.

Initially, the amplified p27, p20 and p18 genes were cloned into the SmaI site of the pUC118 vector. Restriction sites for HindIII and XbaI were included in the sense primers to facilitate subcloning into the pETH expression vector and

the pVF transformation vector once clones with the correct orientation were identified.

Restriction digestion of plasmid DNA. Purification of pUC118 from *E. coli* DH5α was done from 3 ml overnight cultures grown at 37°C and shaking at 180 rpm in 2 x YT medium (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl, pH 7.0) using a modification of the procedure of Sambrook et al. (1989). The only difference was the use of a half volume of 7.5 M ammonium acetate for the precipitation of bacterial proteins prior to the DNA precipitation. The plasmid DNA was resuspended in 30 μl of sterile distilled water.

For each ligation reaction approximately 0.5 μg of pUC118 were digested with SmaI according to the manufacturer's recommendations in a final volume of 30 μl. Digestion was performed for 3 to 4 h, and the DNA was subsequently purified using phenol, phenol: chloroform and chloroform extractions and precipitated with ethanol (Sambrook et al., 1989).

Ligation reaction. For ligation of the PCR products into the SmaI digested pUC118, the purified PCR products (10 μl) were first treated with 5 units of DNA polymerase I (Klenow fragment) in a final volume of 15 μl containing 90 mM Tris-HCl pH 7.5 (at 37°C), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dNTPs. Incubation was for 10 min at room temperature, 20 min at 37°C and 10 min at 70°C. The PCR products were then treated with phenol, phenol: chloroform, chloroform and precipitated with

ethanol (Sambrook et al. 1989). The dried pellets were resuspended in 16 μl of sterile distilled water.

The total volume of recovered PCR products was mixed with the purified SmaI digested pUC118 for ligation in 500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 200 mM DTT, 10 mM ATP, 0.05% bovine serum albumin type V (BSA) and T4 polynucleotide kinase (30 u) in a final volume of 28 μl and incubated at 37°C for 45 min. After that, 2 μl of T4 DNA ligase (6 u) were added to the reaction and incubated overnight at 16°C or, alternatively, incubated at room temperature for 2 h.

Bacterial transformation. After ligation, *E. coli* DH5α competent cells were prepared and transformed using the calcium chloride procedure (Sambrook et al., 1989).

Transformed bacteria were plated on 2 x YT agar medium containing 100 μg/ml of ampicillin and 40 μg/ml of 5-bromo-4-chloro-3-idolyl-β-D-galactoside (X-gal) and incubated overnight at 37°C. White bacterial colonies were transferred to 2 x YT agar plates containing ampicillin for further analysis.

Detection of recombinant plasmids. The rapid disruption of bacterial colonies method was used to determine the presence of recombinant plasmids containing the gene of interest (Sambrook et al., 1989). Plasmid sizes were compared in 0.8% agarose gel electrophoresis to a standard of pUC118 without insert; those with insert migrated slower in the gels.

Recombinant plasmids were then purified from liquid cultures by the procedure of Sambrook et al. (1989). To confirm the presence of an insert and determine its orientation, approximately 0.5 μg of plasmid DNA were digested with 5 u of HindIII following the manufacturer's instructions. Clones with sense orientation in pUC118 were used for subcloning into the expression vector.

Subcloning of CTV ORFs in the Expression Vector

The plasmid pETH-3b (McCarty et al., 1991) was used for expression of the CTV genes p27, p20 and p18. The inserts were recovered from pUC118 by digestion with HindIII and EcoRI. Approximately 5 μg of plasmid DNA were digested with HindIII (10 u) and EcoRI (20 u) in a final volume of 30 μl in 6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT for 3 to 4 h at 37°C. The digestion products were electrophoresed in a 0.8% LMP agarose gel and the inserts purified as described above.

DNA from pETH-3b (0.5 μg per reaction) was also digested with HindIII (2.5 u) and EcoRI (5 u) and the linearized plasmid similarly purified in LMP agarose. Ligation and bacterial transformation into *E. coli* DH5α were as described above, omitting only the addition of DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase. Transformed bacteria were grown in 2 x YT agar media containing 100 μg/ml of ampicillin. Bacterial colonies were screened using the rapid disruption of bacterial colonies method (Sambrook et al.,

1989). Colonies, containing the recombinant plasmid, were replicated for later use.

Plasmid DNA was purified (Sambrook et al., 1989) and transformed into *E. coli* BL21(DE3) (the expression host for pETH plasmids) competent cells prepared by the calcium chloride method (Sambrook et al., 1989). The bacteria were plated on LB agar (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0, 1.5% agar) medium supplemented with 100 μg/ml of ampicillin and 25 μg/ml of chloramphenicol.

DNA Sequencing

The inserts in the recombinant plasmids were sequenced to confirm the presence of the CTV genes and their correct orientation. Plasmid DNA was purified as before from *E. coli* DH5α recombinant colonies grown in 2 x YT liquid cultures with antibiotics (Sambrook et al., 1989). Approximately 2 μg of DNA were used for each labeling reaction. The DNA was first denatured in 20 μl of 1 N NaOH, 25 mM EDTA by incubation at 70°C for 5 min and quickly chilled on ice, followed by ethanol precipitation in 0.3 M sodium acetate (Sambrook et al., 1989). The samples were incubated at -80°C for 30 min before centrifugation for 15 min at 14,000 rpm. The DNA was resuspended in 7 μl of distilled water.

The labeling reaction was performed using the Sequenase Version 2.0 sequencing kit (Amersham) according to the manufacture's instructions. The technique is based on the chain termination method (Sanger et al. 1977). For each

reaction 12.5 μCi of [α-^{35}S]-dATP were used. Both strands of DNA were sequenced using primers specific for the pETH expression vector.

Samples were electrophoresed in denaturing 5% polyacrylamide gels (acrylamide/bisacrylamide 19:1, 1x TBE, 8 M urea) for 1.5 to 4 h at room temperature and 35 W constant power.

Bacterial Expression of CTV Proteins

Induction. The expression of CTV genes in *E. coli* BL21(DE3) was induced using isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.4 M, in 25 ml of LB liquid medium supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol (Sambrook et al., 1989).

To test for expression of recombinant proteins, 1 ml samples were taken before induction and 30 min, 1 h, 2 h and 3 h after induction. All samples were kept on ice in microcentrifuge tubes until the last was collected. Bacterial cells were pelleted by centrifugation at 14,000 rpm for 30 sec at room temperature and resuspended in 50 μl of cracking buffer (300 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 1% v/v 2-mercaptoethanol, 0.025% bromophenol blue). After resuspension, the samples were boiled for 3 min and stored at -20°C for later use.

Detection. Bacterial proteins were separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were electrophoresed at 200 V for 45 min in a

7 x 8 cm gel at room temperature. Protein standards SDS-6 or SDS-7 were included. Proteins were stained using Coomassie brilliant blue G-250 for 30 min (Sambrook et al., 1989).

Production of Polyclonal Antisera

Protein expression. Overnight 3 ml cultures of each recombinant pETH-3b plasmid containing the CTV p27, p20 or p18 ORFs were used for production of recombinant protein. The medium used was LB with antibiotics as described above and incubation was at 37°C with shaking (180 rpm). In the morning, 25 ml of fresh medium were inoculated with 0.5 ml of the overnight cultures and incubated for 2 h. The cultures were then induced with IPTG and incubated for 3 more h. Cells were transferred to a 15 ml tube and centrifuged at 4°C for 10 min and 15,000 g. The bacteria were resuspended in half their original volume in TE buffer and frozen overnight at -20°C. Cells were thawed at 37°C, vortexed for a few minutes and centrifuged as before. Both the supernatant and pellet, were collected the first time, and samples were treated with cracking buffer for analysis in SDS-PAGE to determine the fraction that contains the protein of interest. In those cases in which the protein was insoluble (p27 and p18) the pellet was resuspended in one half volume (6 ml) of TE buffer and pelleted by centrifugation. This was repeated 2 more times, each time reducing the volume of TE buffer in half. The final volume was 1.5 ml.

For p20, most of the protein was soluble. The protein in the supernatant was pelleted with saturated ammonium sulfate (final concentration 33%; Ausubel et al., 1992) and centrifuged 14,500 g for 15 min at 4°C. The pellet was resuspended in TE buffer. All three proteins were stored at -20°C.

Isolation of recombinant proteins. Proteins of interest were separated from other bacterial components using 12% SDS-PAGE in a 15 x 15 cm gel and a single well, 3 mm thick comb. Approximately 5 to 10 mg were separated in each gel. Proteins were detected using ice cold 0.25 M KCl (Hager and Burgess, 1980) which reacted with the bound SDS to form a visible band. The protein band was excised with a sterile blade and stored at 4°C.

Polyclonal antibodies. The protein (still in the polyacrylamide gel) was sent to Cocalico Biological, Inc. (Reamstown, PA) for production of polyclonal antibodies in rabbits. Approximately 1.5 to 2 mg of protein were used for each weekly injection. Antibodies were collected and tested after four weeks of injections.

Detection of CTV Proteins in Infected Citrus Tissue

Western and tissue blot analysis. The expression of p27, p20 and p18 in T36 CTV-infected Mexican lime was tested using Western blot analysis, according to a previously described procedure (Li et al., 1991). Detached midrib segments of approximately 1 cm in length were sliced into small pieces

using a blade and combined with 0.5 ml of cracking buffer, boiled for 3 min and stored at -20°C. Protein extracts (10 μl) were separated by 15% (p27) or 12% (p20 and p18) SDS-PAGE along with the appropriate molecular weight markers. The proteins were transferred to a nitrocellulose membrane using electrophoretic transfer in 25 mM Tris, 192 mM glycine, 20% v/v methanol at 4°C for 1 h at 100 V, 350 mA. Membranes were probed with the different polyclonal antisera using the colorimetric procedure. For p18 a chemiluminescent procedure was also tested using the Renaissance kit. A monoclonal antibody (MCA-13) raised to the T36 CP (Permar et al., 1990) also was used.

Tissue blot analyses also were tested as a rapid method for the detection of p27 and p20 using the procedure of Garnsey et al. (1993). Two incubation conditions (room temperature and 37°C for 1 h) were tested, as well as dilutions of antiserum from 1:500 to 1:20,000.

Cell fractionation. Cell fractions from T36 CTV-infected and uninfected citrus tissue were prepared by a modification of the procedures of Godefroy-Colburn et al. (1986) and Albretch et al. (1988). Approximately 1 g of tissue was ground in liquid nitrogen and thawed in 2 ml of grinding buffer (100 mM Tris-HCl pH 8.1, 10 mM KCl, 0.4 M sucrose, 10% glycerol, 10 mM 2-mercaptoethanol). The mixture was incubated with agitation for 15 min at room temperature and then filtered by centrifugation at 3,000 g for 10 min through a 64-mesh nylon

cloth fused to a 3 ml syringe. Both filtrate and solid residue were collected. The liquid filtrate was centrifuged at 1000 g for 10 min; both pellet (fraction P1) and supernatant were collected. The supernatant was further centrifuged at 30,000 g for 20 min, and the pellet (fraction P30) and supernatant (fraction S30) were collected. The solid residue from the first step was resuspended in 1 ml of grinding buffer and incubated with agitation for 15 min at room temperature. The extract was filtered again by centrifugation. The filtrate (fraction R1) was collected and the solid residue resuspended in 1 ml of grinding buffer containing 2% Triton X-100 for 15 min with agitation and subsequently filtered again by centrifugation. The filtrate (fraction R2) was collected and the solid residue was resuspended in 1 ml of 75 mM Tris-HCl pH 6.8, 4.5% SDS, 9 M Urea, 7.2% 2-mercaptoethanol at room temperature for a final 15 min extraction with agitation, followed by filtration. The filtrate was collected (fraction R3) and the solid residue discarded. All fractions were combined with equal volumes of cracking buffer and boiled for 3 min for analysis by Western blots.

Results

Cloning of CTV Genes

CTV genes for p27, p20 and p18 were amplified by RT-PCR and first cloned into the SmaI restriction site of pUC118 using blunt-end ligation. A HindIII restriction site was

included in the sequence of the sense primer (upstream) to facilitate subcloning in pETH-3b. Identification of the clonal orientation was necessary for subcloning into the pETH vector. Clones with the EcoRI restriction site of the plasmid polylinker located downstream of the gene were used for subcloning. These clones were identified by restriction digestion of the plasmid DNA with HindIII. Clones in the sense orientation were only linearized upon digestion without releasing the inserts, whereas clones in the antisense orientation released the inserts (data not shown).

For subcloning into pETH-3b and to guarantee proper orientation and reading frame of the gene, two non-complementary restriction enzymes that generated cohesive termini were used: HindIII, located 5′ to the start codon and EcoRI, located 3′ to the stop codon of each CTV gene. This strategy also avoided self-ligation of the plasmid. Sequencing of the clones (data not shown) confirmed orientation and reading frame for each construct.

Expression of Proteins

CTV genes were expressed as fusion proteins, made up of 20 amino acids of the T7 coat protein (Studier et al., 1990; McCarty et al., 1991) and the amino acids of each CTV gene. The fusion part of the proteins increased the MW by 1.9 kDa. The resulting predicted MW of each protein was 29.3 kDa (p27), 22.4 kDa (p20) and 20.2 kDa (p18).

Analysis of bacterial proteins by SDS-PAGE showed accumulation of proteins of the expected MW in induced cultures, but not in non-induced cultures (data not shown). The amount of each protein increased with time. CTV proteins accumulated to approximately 30% of the total bacterial protein.

Detection of p27

The expression of p27 in CTV-infected citrus was tested using Western blot analysis. The optimal dilution of antiserum was determined empirically. Best results were obtained at a dilution of 1:500. Because of the amino acid homology between p27 and the CP the reaction of different antisera to both proteins were compared (Figure 2).

The p27 polyclonal antiserum showed some nonspecific reactivity (as did pre-immune serum) with uninfected citrus tissue (Fig. 2, lane 4). However, a discrete, intense, protein band of approximately 27 kDa was detected in extracts of CTV-infected citrus (Fig. 2, lane 5), but not in those of uninfected citrus (Fig. 2, lane 4). The antiserum also detected *E. coli*-expressed p27 (Fig. 2, lane 2), which migrates slightly slower than p27 due to its higher MW. While the p27 polyclonal antibodies reacted with the purified fusion p27 protein (Fig. 2, lane 2), they did not react with a non-fusion, *E. coli*-expressed CP (Fig. 2, lane 3). Furthermore, no reaction was observed with proteins induced from bacteria containing the pETH plasmid without an insert (Fig. 2, lane

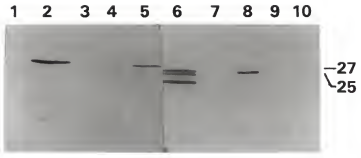


Figure 2. Western blot analysis of CTV-infected and uninfected citrus leaf extracts with p27 polyclonal antiserum (lanes 1-5) and with MCA-13 monoclonal antibody (lanes 6-10). Lanes 1 and 10, pETH-3b without insert; Lanes 2 and 9, *E. coli*-expressed fusion p27 (lane 9 contains 100 times more protein than lane 2); Lanes 3 and 8, *E. coli*-expressed CP; Lanes 4 and 7, uninfected citrus extracts; Lanes 5 and 6, CTV T36-infected citrus extracts. Molecular weights in kilodaltons are indicated on the right.

1). Finally, no p27 band was detected when preimmune serum was used (data not shown), indicating the specificity of the antibody.

To preclude the possibility that the protein detected in infected tissue was a stress-induced pathogenesis-related protein, tissue extracts from citrus plants infected with citrus ringspot virus, citrus variegation virus, citrus leaf rugose virus, psorosis A and concave gum also were analyzed by Western blot, using p27 antiserum. No p27 band was observed in any of the samples (data not shown).

To further demonstrate the specificity of the antibody, half of the same membrane in Fig. 2 (lanes 6 to 10) was probed with MCA-13. This monoclonal antiserum, specific to the CP, reacted strongly with the *E. coli*-expressed CP (Fig. 2, lane 8) and with the CP in the extract of CTV-infected citrus tissue (Fig. 2, lane 6), including the lower molecular weight proteolysis products of the CP (Sekiya et al., 1991). No reactions were observed with proteins produced by bacteria containing the pETH plasmid without an insert (Fig. 2, lane 10) or from extracts of uninfected citrus tissue (Fig. 2, lane 7).

Interestingly, MCA-13 gave a weak but consistent reaction with the purified p27 fusion protein, which could be seen only on the membrane (not visible in Fig. 2, lane 9), but not with p27 from infected tissue. However, to obtain this level of reaction with MCA-13, it was necessary to increase the

concentration of p27 protein by 100-fold over the amount used in Fig. 2, lane 2, where it was readily detected by the p27 antiserum.

To determine the cellular localization of p27, cell fractions from CTV-infected and uninfected citrus tissue were prepared and assayed by Western blots. Similar percentages of total protein for each fraction were analyzed using p27 antiserum (Fig. 3). All cell fractions contained p27. However, most of the p27 accumulated in the cell wall fractions (R1, R2 and R3) (Fig. 3, lanes 6 to 8, respectively). In addition, fraction S30 also contained considerable amounts of p27 (Fig. 3, lanes 5). Fractions P1 and P30 showed much lower levels of p27 (Fig. 3, lanes 3 and 4, respectively). These results indicate that p27 was mostly associated with the cell wall fractions.

The reaction of MCA-13 to the cell fractions was also tested to compare the accumulation of CP and p27. Fig. 4 indicates that most of the CP accumulated in the cell wall fractions R1 and R2 (Fig. 4, lanes 6 and 7), with some also present in the soluble protein fraction S30 (Fig. 4, lane 5). Fractions P1 and P2 contained less CP (Fig. 4, lanes 3 and 4). The pattern of accumulation of the CP was similar to that observed for p27.

The p27 antiserum also was tested using tissue blot (Fig. 5). The optimal conditions for specific detection were

Figure 3. Western blot analysis of cell fractions prepared from CTV-infected tissue and probed with p27 antiserum. Lane 1, uninfected citrus tissue extract; Lane 2, unfractionated infected citrus extract; Lanes 3 to 8 contained fractions P1, P30, S30, R1, R2 and R3, respectively, of infected citrus tissue as explained in Materials and Methods. Lane 9 is *E. coli*-expressed p27.

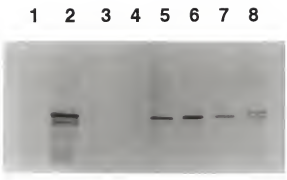


Figure 4. Western blot analysis of cell fractions prepared from CTV-infected tissue and probed with MCA-13 monoclonal antibody. Lane 1, uninfected citrus tissue extract; Lane 2, unfractionated infected citrus extract; Lanes 3 to 8 contained fractions P1, P30, S30, R1, R2 and R3, respectively, of infected citrus tissue as explained in Materials and Methods.

A

B

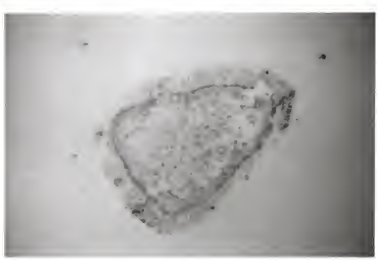


Figure 5. Tissue blots probed with the p27 polyclonal antiserum. A, CTV-infected citrus tissue; B, uninfected citrus tissue.

incubation at 37°C for 1 h, and 1:10,000 dilution. The blots indicate that p27 is present in phloem tissue.

Detection of p20

The optimal antiserum concentration for the analysis of p20 was 1:500. Fig. 6 shows the results of the detection of the cell fractions with p20 antiserum. A protein band of the expected size was present in infected, unfractionated tissue (Fig. 6, lane 2) but not in uninfected tissue (Fig. 6, lane 1). Most of the p20 was in the soluble protein fraction (S30) (Fig. 6, lane 5). Cell wall fraction R3 (Fig. 6, lane 8) also contained some p20. Fractions P1, P30, R1 and R2 contained little or no detectable p20 (Fig 6, lanes 3, 4, 6 and 7, respectively).

The p20 antiserum was not useful for detection of the protein using tissue blots. At high antiserum concentrations (1: 1000) the serum was reactive with uninfected tissue, and at higher dilutions (1: 20,000) positive reactions with CTV-infected tissue were very weak.

Detection of p18

Despite several attempts to detect p18 in infected tissue, no band of the expected size was observed in Western blots. Dilution of the antiserum as low as 1:100 did not produce any favorable results in colorimetric detection (data not shown). The antibody reacted, however, with fusion p20 produced in bacteria, the same protein used as antigen (data not shown). It also reacted with fusion p18 and p27, both of

Figure 6. Western blot analysis of cell fractions prepared from CTV-infected tissue and probed with p20 antiserum. Lane 1, uninfected citrus tissue extract; Lane 2, unfractionated infected citrus extract; Lanes 3 to 8 contained fractions P1, P30, S30, R1, R2 and R3, respectively, of infected citrus tissue as explained in Materials and Methods. Lane 9 is *E. coli*-expressed p20.

which contained the same 20 N-terminal amino acids. Detection of cell fractions with the antiserum did not reveal any bands of the expected MW. A more sensitive, chemiluminescent detection system was also used without positive results (data not shown).

Discussion

The levels of bacterial expression obtained with the fusion proteins were similar to those previously reported (Marston, 1986; Studier et al., 1990). With this expression system, it was possible to successfully produce the three CTV proteins and purify them by a relatively simple method. The antisera produced reacted with the antigens and gave low non-specific reactions when used to probe tissue samples. There was variation in the solubility of the fusion proteins, with p27 and p18 accumulating as insoluble products, and p20 being soluble. This did not affect the levels of expression or the isolation of the proteins.

The presence of the fusion portion seemed to stabilize some of the proteins in *E. coli*. Attempts to express p27 without this portion rendered very low levels of the protein (data not shown). However, nonfusion CP was expressed in the same bacterial host (K.L. Manjunath, personal communication). The only inconvenience with fusion proteins is that antisera produced to different fusion proteins cross react with all the antigens, since the fusion part is identical. This might limit the use of the recombinant proteins as positive controls,

although it does not have any effect on the detection of the target proteins *in vivo* (because they lack the fusion portion).

The results using the p27 antiserum showed the presence of a protein of the expected size (27.4 kDa) in CTV-infected, but not in uninfected tissue. Also, this protein band was not present in tissue samples infected with other citrus viruses. This indicates that the protein detected is CTV-specific and was expressed during infection, and that its size is in agreement with that predicted from the sequencing data (Pappu et al., 1994).

The protein bands detected with the CP-specific monoclonal antibody MCA-13 are of a lower MW than that detected with p27 antiserum. Because of this and the fact that the p27 antiserum detected only a single band, and did not react with the nonfusion *E. coli*-expressed CP, it is concluded that the protein detected with the antiserum is p27, and not the result of a cross-reaction of the p27 antiserum with the CP. This was a definite possibility since both proteins have a high degree of homology in their primary structure.

The monoclonal antibody MCA-13 showed a very low affinity for the *E. coli*-expressed p27, because its concentration had to be increased 100-fold compared to the concentration of p27. The region of the CP that is recognized by MCA-13 has been identified (Pappu et al., 1993a) and part of this region is conserved in p27, which may explain its low affinity for p27.

The tissue fractionation experiments showed that p27 and the CP are mostly associated with the cell wall and soluble protein fractions. Conversely, in BYV, p24 (the CP homologue) and the CP were shown to accumulate mostly in the cytoplasm or soluble protein fraction (Agranovsky et al., 1994). Comparison of the sequences upstream of the start codons and surrounding the initiation sites for the subgenomic RNAs of BYV p24 and CP revealed a consensus sequence, (Agranovsky et al., 1994), suggesting concerted expression of both genes. This sequence was similar to those found in the CPs of tobamoviruses, brome mosaic virus, cucumber mosaic virus and alfalfa mosaic virus (AlMV; Agranovsky et al., 1994). Comparison of the sequences upstream to the start codons of CTV p27 and CP did not revealed any consensus sequences similar to those of BYV or LIYV (data not shown). This indicates that the expression of these two set of genes is different in CTV and BYV.

Recently, p24 was found to be part of the BYV virions, forming a terminal "tail" at one end of the particles (Agranovsky, 1995). Due to the similarities between the two viruses and the apparent structural conservation between the CP homologues (Pappu et al., 1994), it is possible that p27 may also form part of the CTV virions. This is currently under investigation using the p27 specific antiserum.

The function of p27 in CTV is still not known. Besides its possible structural role, if p27 forms part of the virions, it might also be involved in particle assembly

(Agranovsky et al., 1995). This could be through interaction with other viral proteins, including the CP and the heat shock proteins (p65 and p61) or unknown host components. Heat shock proteins work as chaperons helping in proper folding of other proteins (Georgopoulos, 1992).

The p27 might also have a role as a helper component in the transmission of CTV by its aphid vector. This has previously been proposed for BYV p24 (Agranovsky et al., 1995; Boyko et al., 1992). Helper components have been described for potyviruses and caulimoviruses (Mathews, 1991), although they do not form part of the virions. However, in potyviruses the CP is also important for aphid transmission (Atreya et al., 1990, 1991). Interestingly, LIYV also contains a similar duplicate of the CP, with deduced amino acid sequence homologies to BYV and CTV CPs and homologues, however, LIYV is transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Klaassen et al., 1995).

Relating to the cellular accumulation of p27, a possible function could be as a movement protein, assisting in the spread of the virus between cells. Movement proteins interact with plasmodesmata causing an increase in their diameter (Wolf et al., 1989; Derrick et al., 1992; Waigmann et al., 1994), thereby enabling the intercellular passage of the large nucleic acid in a naked or coated form, depending on the virus. Putative movement proteins of tobacco mosaic virus (Deom et al., 1987), AlMV (Godefroy-Colburn et al., 1986),

cauliflower mosaic virus (Albrecht et al., 1988) and squash leaf curl geminivirus (Pascal et al., 1993) accumulate in the cell wall fractions. The observations that p27 also accumulates in this fraction suggest a possible role in virus movement.

The capacity to bind RNA is another characteristic of movement proteins (Deom et al., 1987; Citovsky et al., 1992; Giesman-Cookmeyer and Lommel, 1993). Due to the apparent structural similarities between p27 and the CP (Pappu et al., 1994), including the conservation of amino acids present in the CPs of other filamentous viruses (Dolja et al., 1991; Boyko et al., 1992), and the finding of p24 as part of the BYV virions, it is likely that p27 might have RNA-binding capacity.

The CP of some RNA viruses also have a role in cell-to-cell and long distance movement. In some cases, like cowpea mosaic comovirus (van Lent et al., 1991), the virus moves from cell to cell as virions. In other cases, like AlMV, the CP is required for movement, but virions are not transported (van der Vossen et al., 1994). For brome mosaic bromovirus, the CP is not required for cell-to-cell movement, but is required for long distance movement (Flasinski et al., 1995). It is possible then that p27 acts as a multifunctional protein, forming part of the virus particles and assisting in one or more of the following functions: virus movement, aphid transmission or encapsidation.

Using tissue blots, p27 was mostly present in the sieve tubes. This is in agreement with what has been found for the CP (Garnsey et al., 1993) and with the phloem-limited nature of CTV (Bar-Joseph and Lee, 1990). These results also indicate that some of the p27 epitopes are conserved between native and SDS-denatured protein. The antiserum was raised against denatured protein, however, in tissue blots the proteins are not treated with SDS previous to their detection.

Tissue blot is a simple method for assaying the expression of p27, especially when a large number of samples are involved. This technique will be used for the screening of the p27 transformed plants.

The polyclonal antiserum raised using recombinant p20 detected a protein band of the expected size in CTV-infected, but not in uninfected citrus tissue, indicating that the p20 also is CTV specific. Unlike p27, p20 accumulates in the soluble protein fraction. The intensity of the p20 band in Western blots was much lower for p20 than p27. This was not expected since analysis of CTV subgenomic RNAs indicates that p20 transcript is the most abundant (Hilf et al., 1995). One alternative to explain this result is that p20 protein is poorly immunogenic, and so the concentration of specific antibodies in the serum is low. Because the *E. coli*-expressed p20 contains the fusion portion, it is not possible to conclude anything from the reaction observed with the antiserum to this protein. Another possibility is that p20

protein expression is regulated post-transcriptionally and that its RNA plays some role in the virus life cycle.

Regarding the function of p20, it is still difficult to speculate since the protein does not show any similarities to previously described proteins. However, this research demonstrates that the ORF is expressed during CTV infection.

The p18 protein was not detected in CTV-infected tissue samples using Western blot analysis, even when the more sensitive chemiluminescent method was used. The antiserum, however, reacted with *E. coli*-expressed p18. Subgenomic RNA analysis indicates that p18 is expressed at a very low level compared to p27, CP or p20 (Hilf et al., 1995). Low protein expression and/or expression at a specific time during the CTV life cycle may explain the inability to detect p18.

CHAPTER 3
SEQUENCE ANALYSIS OF THE P27 ORF

Introduction

The p27 ORF has been described as a diverged copy of the CP gene (Pappu et al., 1994). Comparison of the deduced amino acid sequences indicates that 19% of the amino acids are identical between p27 and the CP, and 41% of the amino acids are either identical or with similar biochemical properties to those of the CP (Pappu et al., 1994). The first report of the diverged copy of the CP among closteroviruses (and filamentous RNA viruses) was for BYV (Boyko et al., 1992). The BYV p24 ORF also is located 5' to the CP and all four genes of CTV and BYV show various degrees of amino acid sequence similarities, ranging from 38% (CTV p27 and BYV CP) to 50% (CTV p27 and BYV p24) (Boyko et al., 1992; Pappu et al., 1994). Two of the amino acids conserved in the four proteins also are strictly conserved in the CPs of 14 other filamentous positive-stranded RNA viruses (Dolja et al., 1991; Boyko et al., 1992; Pappu et al., 1994). Those two residues are believed to form a salt bridge (Dolja et al., 1991), suggesting that the CPs and the diverged copies share not only the primary structure, but exhibit similar protein folding (Boyko et al., 1992).

Detection of sequence variability in viral genes is important since mutations could be linked to particular biological properties. Single stranded conformation polymorphism (SSCP) is a simple and sensitive method used to detect single nucleotide changes in particular sequences (Orita et al., 1989). This technique is based on migration differences during electrophoresis of short, denatured, DNA fragments in non-denaturing gels, caused by differences in their nucleotide sequences (Orita et al., 1989; Spinardi et al., 1991).

The main purpose of this research was to determine if there was any relationship between RNA or deduced protein sequence and the biological properties of the virus. Therefore, this gene was cloned and sequenced from CTV strains with distinct geographical origins and different biological activities. In addition, PCR combined with SSCP analysis was tested for its potential as a rapid method to determine sequence differences among CTV strains.

Materials and Methods

Materials

In addition to the materials mentioned in Chapter 2, the Wizard DNA purification system used was from Promega Corporation (Madison, WI) and the silver staining kit was from Sigma Chemical Company (St. Louis, MO). Sequence analysis was performed using the programs Seqaid, CLUSTALV (Higgins et al.,

1992) and Wisconsin Genetic Computer Group Sequencing Analysis Software (GCG) version 7.0 (Devereux et al., 1984) provided by the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL).

Virus Isolates

All CTV strains (except for T30 and T36) were obtained from the Exotic Citrus Pathogens Collection, maintained by the USDA and IFAS in Beltsville, MD. Strains T30 and T36 were maintained in greenhouses at the University of Florida (Gainesville, FL). Propagation hosts for the strains were "Madam Vinous" sweet orange [*C. sinensis* (L.) Osbeck] or "Mexican" lime [*C. aurantifolia* (Christm.) Swingle].

Cloning and Sequencing

Biological properties of the strains used in sequencing are listed in Table 2. Cloning and sequencing procedures were similar to those described in Materials and Methods, Chapter 2. In some cases (strains B7-1, B185, B227 and B249) PCR products and plasmid DNA were purified using the Wizard DNA Clean-Up System. For B50 and B67, both nucleic acid extracts and dsRNA (Morris and Dodds, 1979) were used for PCR amplification. When dsRNA was used, denaturation was performed in a solution containing 10 mM dimethylmercury and approximately 100 pM of each primer in a total volume of 10 μl, incubated at room temperature for 8 min and then quick frozen in liquid nitrogen. The mixture was then used in a standard PRC reaction as described in Chapter 2.

Table 2. Characteristics of the CTV strains used for sequencing of the p27 gene.

STRAIN	ORIGIN	MCA13[a]	M.L.[b]	DECLINE[c]	SY[d]	SP-G[e]	SP-MV[f]	COMMENTS
T26	Florida	-	+	0	0	0	0	
T30	Florida	-	+	0	0	0	0	Effective for cross protection
T36	Florida	+	++	++	+	+	0	Genome sequenced
B7-1	S. Africa	+	++	+	+	++	0	Used for cross protection.
B67	China	-	+	0	0	0	0	
B128	Colombia	+	++	?	0	+++	+	
B185	Japan	+	+++	+++	+++	+++	++	
B227	India	+	++	+++	++	+++	+++	
B249	Venezuela	+	++	+++	+++	++	+	Vein corking on M.L.

a MCA13= Reactive with the monoclonal antybody MCA13 (-= no reaction, += positive reaction).
b M.L.= Symptoms on Mexican lime (+= mild, ++= moderate, +++= severe, ?= unknown).
c Decline= Induces decline in sweet orange scions grafted on sour orange rootstock.
d SY= Seedling yellows on sour orange seedlings.
e SP-G= Stem pitting on grapefruit scions.
f SP-MV= Stem pitting on Madame Vinous sweet orange scions.

In addition to the p27 genes, the CP from strains B249 was also cloned and sequenced. Sequences for all the other CP genes were kindly provided by H.R. Pappu. Clones for the p27 gene of isolate B128 were kindly provided by S.S. Pappu.

At least two clones of each strain were sequenced in both the sense and antisense directions. Sequences were compared using the programs CLUSTALV and GCG. Deduced amino acid sequences were obtained using Seqaid.

Single Stranded Conformation Polymorphism (SSCP)

The same strains employed for sequencing were also used for SSCP analysis. Other strains are described in Table 3. The p27 genes were amplified from tissue extracts or from plasmid DNA and analyzed in non-denaturing polyacrylamide gels. PCR products (2 μl) were denatured in 95% formamide, 20 mM EDTA, 0.05% bromophenol blue by incubation at 100°C for 10 min and electrophoresed in 7 x 8 cm, 8% polyacrylamide gels (acrylamide: bisacrylamide 30:1, 1 x TBE) for 3 hrs 45 min at 200 V in 1x TBE buffer at room temperature. The buffer and gels were pre-chilled at 4°C overnight before electrophoresis. DNA was detected using silver staining.

Results

Sequencing

The CTV strains sequenced were selected based on diversity of biological properties and geographical origins. Table 4 summarizes the overall similarities of nucleotide and

Table 3. Characteristics of the CTV strains used for SSCP analysis.

STRAIN	ORIGIN	MCA-13[a]	M.L.[b]	DECLINE[c]	SY[d]	SP-G[e]	SP-MV[f]	COMMENTS
B5	Calif.	-	+	0	0	0	?	
B11	Hawaii	+	+	+	+	0	0	
B28	Florida	+	++	++	++	++	++	
B32	Spain	-	+	0	0	0	0	
B37	Taiwan	+	++	+++	+++	?	+	
B148	Florida	+	++	++	++	0	0	
B192	France	+	0	0	0	0	0	Not aphid transmitted
B272	Colombia	-	0	0	0	0	0	
B274	Colombia	-	+	0	0	0	0	

a through f, as in Table 2.

deduced amino acid sequences among all the strains studied. At the nucleotide level, homology of the sequences varied from 86.2% to 99.6%. Strains B7-1 (severe from South Africa) and B128 (severe from Colombia) were the most similar. At the protein level, homologies were 93.3% or higher. Strains T30 (mild from Florida) and B67 (mild from China) were identical in deduced amino acid sequence. Strains B128 and B249 (severe from Venezuela) also were identical in deduced amino acid sequence.

Alignment of the nucleotide sequences (Fig. 7) showed several nucleotide substitutions along the genes. No deletions, insertions or inversions were observed. All sequences constituted an ORF similar to the one previously reported (Pappu et al., 1994). A total of nine substitutions were conserved in all the severe strains compared to nucleotides conserved in all mild strains. For example, nucleotides 108 (C → T) and 229 (G → A). Other nine substitutions were present only in stem pitting strains. For example, nucleotide 78 (T → C). Most of these substitutions, however were silent.

Comparison of the deduced amino acid sequences (Fig. 8) showed only 4 amino acids exclusive to severe strains (amino acids 9, 77, 104 and 125),and only one in stem pitting strains (amino acid 102). The C-terminal portion of the protein is the most conserved among the strains sequenced.

Table 4. RNA and deduced amino acid sequence similarities (% of identical bases or amino acids) between different CTV strains for p27 (upper right is RNA, lower left is amino acid sequences). Values generated using CLUSTALV.

STRAIN	T30	T26	B67	T36	B7-1	B128	B185	B227	B249
T30		98.9	98.2	93.9	92.5	92.1	90.7	86.2	92.1
T26	98.8		98.3	93.8	91.2	91.4	90.2	88.2	91.6
B67	100.0	98.8		94.6	92.5	92.7	90.3	88.8	92.7
T36	96.7	95.4	96.7		92.5	92.7	91.0	87.6	92.4
B7-1	96.7	95.8	96.7	97.5		99.7	98.1	87.3	99.3
B128	97.1	95.8	97.1	97.9	99.6		98.3	87.6	99.6
B185	94.6	93.3	94.6	95.4	97.1	97.5		86.2	99.6
B227	95.0	93.8	95.0	95.4	96.7	97.1	94.6		87.7
B249	97.1	95.8	97.1	97.9	99.6	100.0	97.5	97.1	

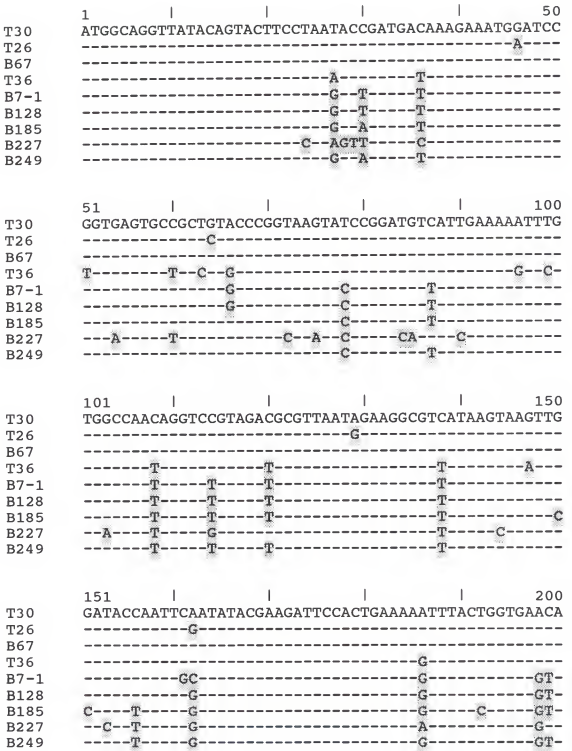
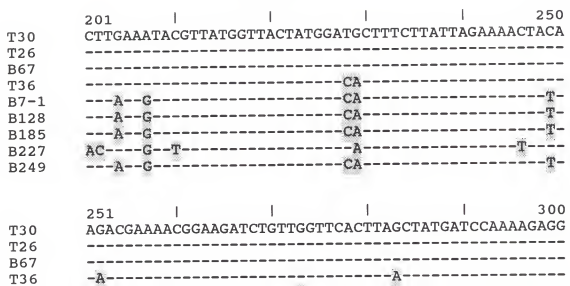
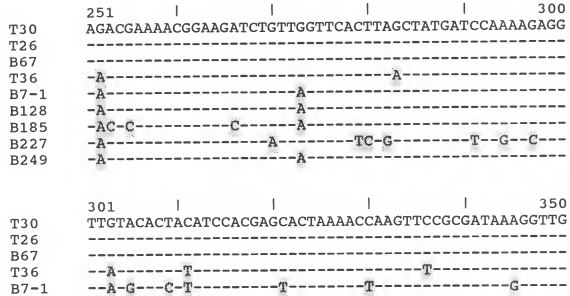
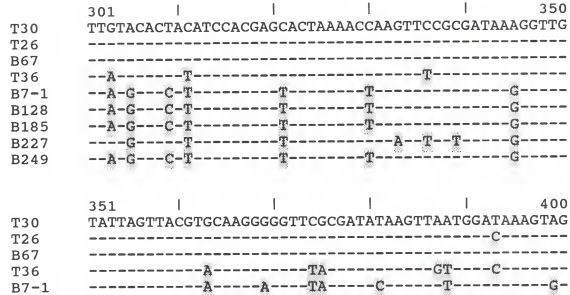


Fig. 7. Nucleotide sequence alignment of p27 gene from different CTV strains (hyphens, identical nucleotides). The alignment was constructed using the program CLUSTALV.







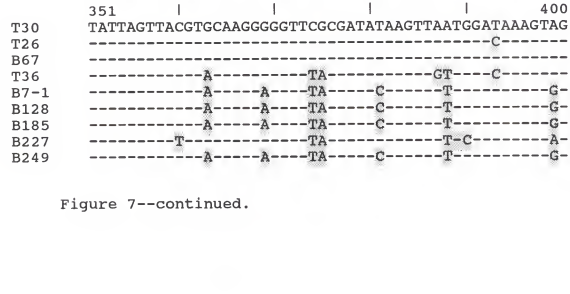


Figure 7--continued.

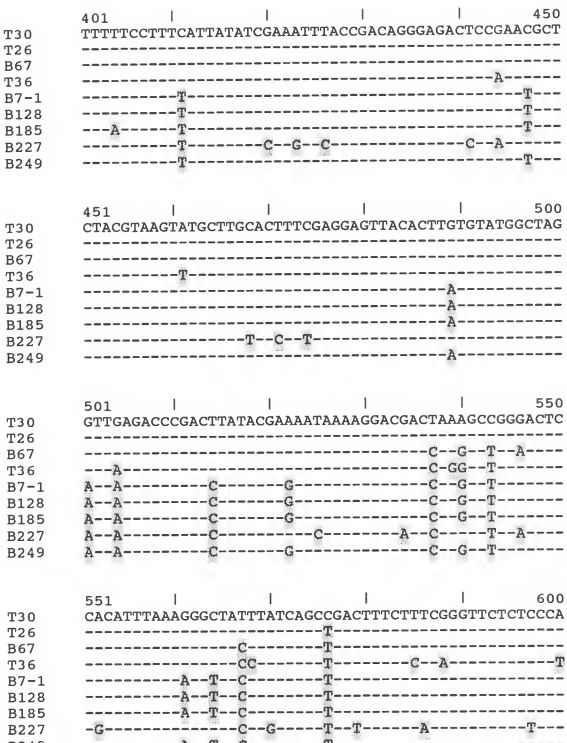


Figure 7--continued.

```
          601       |         |         |         |       650
T30       GGGTACTCCGAACATGAACGAGGCATCATTCTTCGAGCGTCTGAGTCTAT
T26       ----------------------T----------------------------
B67       ----------------------------------------------------
T36       ----------------------T---------------C------------
B7-1      --------T--G----------T-------------C--A------------
B128      --------T--G----------T-------------C--A------------
B185      ------A-T--G----------T-------------C--A------------
B227      --A----------C-----G--T-------------------A---------
B249      --------T--G----------T-------------C--A------------

          651       |         |         |         |       700
T30       GTTAGCTAGACGTCAAGGTTACGAGGAGGCAACCGAGCTTCTTAACCTAC
T26       ----------------------------G-----------------------
B67       ----------------------------------------------------
T36       ----------------------------G-----------------------
B7-1      ----------------------------------------------------
B128      ----------------------------------------------------
B185      ----------------------------------------------------
B227      ----------------------------G---------------T-------
B249      ----------------------------------------------------

          701       |       723
T30       GTGATTTGGGTAAGTACTTATAG
T26       -----------------------
B67       -----------------------
T36       -C---------------------
B7-1      -----------------------
B128      -----------------------
B185      -----------------------
B227      -C---------------------
B249      -----------------------
```

Figure 7--continued.

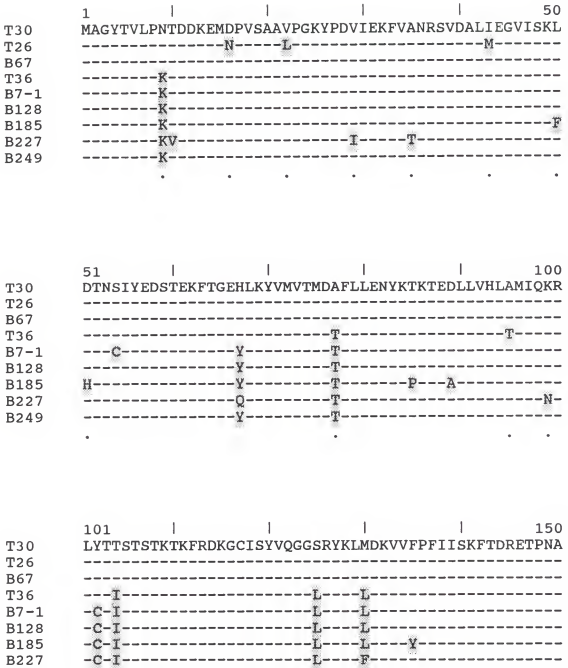


Figure 8. Pairwise alignment of the deduced amino acid sequence of p27 from different CTV strains (hyphens, identical amino acids; periods, similar amino acids). The alignment was constructed using the program CLUSTALV.

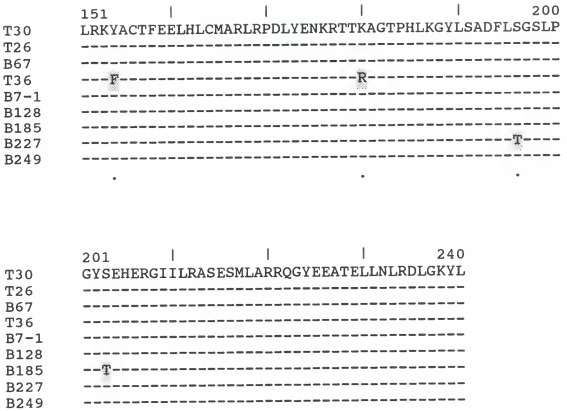


Fig. 8--continued.

A phylogenetic tree was constructed using the RNA sequences (Fig. 9). Four major groups could be distinguished: 1)severe, stem pitting strains B7-1, B128, B249 and B185, 2) mild strains T30, B67 and T26, 3) severe, quick decline strain T36 and 4) severe, stem pitting strain B227. Mild strains showed some relationship to severe, quick decline and stem pitting strains. Strain B227 (severe from India) was the most distinct and formed a completely separate group. The phylogenetic tree constructed using deduced amino acid sequences (Fig. 10) separated mild from severe strains, each one forming a distinct group.

Comparison of the CP sequences in phylogenetic trees (RNA and amino acid, Figs. 11 and 12, respectively) showed a different grouping of the strains from that of p27.

Sequence alignment of p27 and CP from the different strains is showed in Fig. 13. Approximately 20% of the amino acids are identical between the two proteins, depending on the strain. Again, the C-terminal regions of the proteins are also the most conserved (Fig. 13).

Single Stranded Conformation Polymorphism

SSCP analysis of the strains used for sequencing (Fig. 14) revealed five groups with similar patterns: 1) severe, stem pitting strains B7-1 and B128, 2) severe, stem pitting strains B249 and B185, 3) mild strains T30, T26 and B67, 4) quick decline strain T36 and 5) severe, stem pitting strain B227. When all strains were considered (Figs. 14 and 15) they

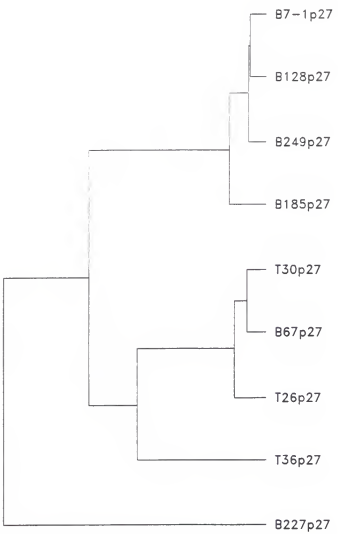


Figure 9. Phylogenetic tree for p27 based on RNA sequences. Graphic generated using Pileup program in GCG.

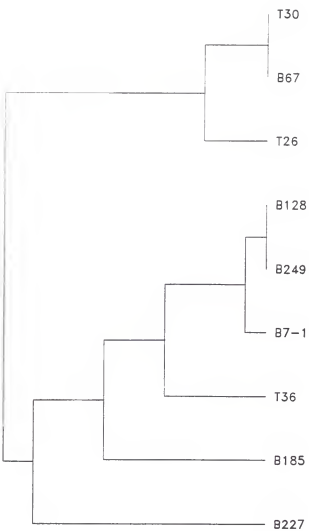


Figure 10. Phylogenetic tree for p27 based on deduced amino acid sequences. Graphic generated using Pileup program in GCG.

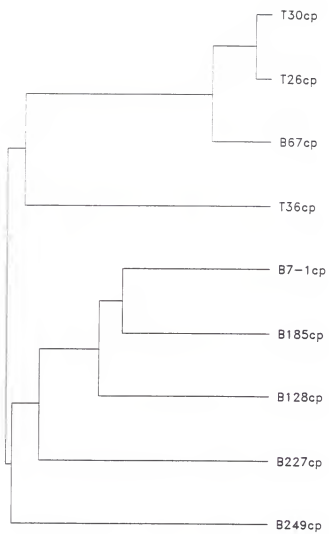


Figure 11. Phylogenetic tree for CP based on RNA sequences. Graphic generated using Pileup program in GCG.

Figure 12. Phylogenetic tree for CP based on deduced amino acid sequences. Graphic generated using Pileup program in GCG.

```
CP T30     MDDETKKLKNKNKETKEGDEVVAAESSFGSVNLHIDPTLITMND--VRQL
CP T26     MDDETKKLNNKNKETKEGDEVVAAESSFGSVNLHIDPTLITMND--VRQL
CP B67     MDDETKKLKNKNKEIKQGDDVVAAESSFGSVNLHIDPTLITMND--VRQL
CP T36     MDDETKKLKNKNKETKEGDDVVAAESSFSSVNLHIDPTLITMND--VRQL
CP B7-1    MDDETKKLKNKNKETKEGDDVVAAESSFGSVNLHIDPTLIAMND--VRQL
CP B128    MDDETKKLKNKNKEAKEGDDVVAAESSFGSLNFHIDPTLIAMND--VRQL
CP B185    MDDETKKLKNKNKETKEGDDVVAAESSFGSLNLHIDPTLIAMND--VRQL
CP B227    MDDETKKLKNKNKETKEGDDVVAAESSFGSMNLHIDPTLIAMND--VRQL
CP B249    MDDETKKLKNKNKETKEGDDVVAAESSFGSLNLHIDPTLIAMND--VRQL
p27 T30    MAGYTVLPNTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKL
p27 T26    MAGYTVLPNTDDKEMNPVSAALPGKYPDVIEKFVANRSVDALMEGVISKL
p27 B67    MAGYTVLPNTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKL
p27 T36    MAGYTVLPKTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKL
p27 B7-1   MAGYTVLPKTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKL
p27 B128   MAGYTVLPKTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKL
p27 B185   MAGYTVLPKTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKF
p27 B227   MAGYTVLPKVDDKEMDPVSAAVPGKYPDIIEKFVTNRSVDALIEGVISKL
p27 B249   MAGYTVLPKTDDKEMDPVSAAVPGKYPDVIEKFVANRSVDALIEGVISKL
           * . *   .  .**       ...  .    ..  . .. .. .  .  . ..

CP T30     STQQNAALNRDLFLALKGKYPNLP----------DKDKDF--HIAMMLYR
CP T26     STQQNAALNRDLFIALKGKYPNLP----------DKDKDF--HIAMMLYR
CP B67     STQQNAALNRDLFLTLKGKYPNLP----------DKDKDF--HIAMMLYR
CP T36     STQQNAALNRDLFLTLKGKHPNLP----------DKDKDF--RIAMMLYR
CP B7-1    STQQNAAVNRDLFLTLKEKYPKLS----------DKDKDF--HIAMMLYR
CP B128    STQQNAALNRDLFLTLKGKYPNLS----------DKDKDF--HLAMMLYR
CP B185    STQQNAALNRDLFLTLKGKYPNLS----------DKDKDF--HLAMMLYR
CP B227    GTQQNAALNRDLFLTLKGKYPNLP----------DKDKDF--HIAMMLYR
CP B249    GTQQNAALNRDLFLTLKGKYPNLP----------DKDKDF--HIAMMLYR
p27 T30    DTNSIYEDSTEKFTGEHLKYVMVTMDAFLLENYKTKTEDLLVHLAMIQKR
p27 T26    DTNSIYEDSTEKFTGEHLKYVMVTMDAFLLENYKTKTEDLLVHLAMIQKR
p27 B67    DTNSIYEDSTEKFTGEHLKYVMVTMDAFLLENYKTKTEDLLVHLAMIQKR
p27 T36    DTNSIYEDSTEKFTGEHLKYVMVTMDTFLLENYKTKTEDLLVHLTMIQKR
p27 B7-1   DTNCIYEDSTEKFTGEYLKYVMVTMDTFLLENYKTKTEDLLVHLAMIQKR
p27 B128   DTNSIYEDSTEKFTGEYLKYVMVTMDTFLLENYKTKTEDLLVHLAMIQKR
p27 B185   HTNSIYEDSTEKFTGEYLKYVMVTMDTFLLENYKPKTEALLVHLAMIQKR
p27 B227   DTNSIYEDSTEKFTGEQLKYVMVTMDTFLLENYKTKTEDLLVHLAMIQNR
p27 B249   DTNSIYEDSTEKFTGEYLKYVMVTMDTFLLENYKTKTEDLLVHLAMIQKR
            *.     . . *    *   .                *   .  ...*.  *
```

Figure 13. Alignment of CPs and p27 from different CTV strains. Asterisks, identical amino acids; periods, similar amino acids. Alignment generated using CLUSTALV.

```
CP T30     LAVKSSSLQSD-DDTTGITYTREGVEVDLSDKLWTDIVYNSKGIGNRTNA
CP T26     LAVKSSSLQSD-DDTTGITYTREGVELDLSDKLWTDIVYNSKGIGNRTNA
CP B67     LAVKSSSLQSD-DDTTGITYTREGVEVDLSDKLWTDVVYNSKGIGNRTNA
CP T36     LAVKSSSLQSD-DDATGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNA
CP B7-1    LAVKSSSLQSD-DDTTGITYTREGVEVDLSDKLWTDVVFNSKGIGNRRNA
CP B128    LAVKSSSLQSD-DDTTGVTYTREGVEVDLSDKLWTDVVFNSKGIGNRTNA
CP B185    LAVKSSSLQSD-DDTTGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNA
CP B227    LAVKSSSLQSD-DDTTGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNA
CP B249    LAVKSSSLQSD-DDTTGVTYTREGVEVELSDKLWTDVVFNSKGIGNRTNA
p27 T30    LYTTSTSTKTKFRDKGCISYVQGGSRYKLMDKVVFPFIISKFTDRETPNA
p27 T26    LYTTSTSTKTKFRDKGCISYVQGGSRYKLMDKVVFPFIISKFTDRETPNA
p27 B67    LYTTSTSTKTKFRDKGCISYVQGGSRYKLMDKVVFPFIISKFTDRETPNA
p27 T36    LYTISTSTKTKFRDKGCISYVQGGLRYKLLDKVVFPFIISKFTDRETPNA
p27 B7-1   LCTISTSTKTKFRDKGCISYVQGGLRYKLLDKVVFPFIISKFTDRETPNA
p27 B128   LCTISTSTKTKFRDKGCISYVQGGLRYKLLDKVVYPFIISKFTDRETPNA
p27 B185   LCTISTSTKTKFRDKGCISYVQGGLRYKLLDKVVFPFIISKFTDRETPNA
p27 B227   LCTISTSTKTKFRDKGCISYVQGGLRYKLFDKVVFPFIISKFTDRETPNA
p27 B249   LCTISTSTKTKFRDKGCISYVQGGLRYKLLDKVVFPFIISKFTDRETPNA
           *  *.* ..   *  ..* . *   * **.  . .    .  **

CP T30     LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP T26     LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP B67     LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP T36     LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP B7-1    LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP B128    LRVWGRSNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP B185    LRVWGRSNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP B227    LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
CP B249    LRVWGRTNDALYLAFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGA--
p27 T30    LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
p27 T26    LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
p27 B67    LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
p27 T36    LRKFACTFEELHLCMARLRPDLYENKRTTRAGTPHLKGYLSADFLSGSLP
p27 B7-1   LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
p27 B128   LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
p27 B185   LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
p27 B227   LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLTGSLP
p27 B249   LRKYACTFEELHLCMARLRPDLYENKRTTKAGTPHLKGYLSADFLSGSLP
           **  . . . * *   *   .*    *   * **  *    ** ****.*.

CP T30     GLTDLECAVYIQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
CP T26     GLTDLECAVYIQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
CP B67     GLTDLECAVYIQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
CP T36     GLTDLECAVYIQAKEQLLKKRGA--DDVVVTNVRQLGKFNTR
CP B7-1    GLTDLECAVYIQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
CP B128    GLTDLECAVYIQAKEQLLKKRGA--DEIVVTNVRQLGKFNTR
CP B185    GLTDLECAVYVQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
CP B227    GLTDLECAVYIQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
CP B249    GLTDLECAVYLQAKEQLLKKRGA--DEVVVTNVRQLGKFNTR
p27 T30    GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 T26    GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 B67    GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 T36    GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 B7-1   GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 B128   GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 B185   GYTEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 B227   GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
p27 B249   GYSEHERGIILRASESMLARRQGYEEATELLNLRDLGKYL--
           * .. * .. ..* * .* .* .  .   . *.*.***.
```

Figure 13--continued.

Figure 14. SSCP analysis of the p27 gene from the same strains used in sequencing. 1, B227; 2, T36; 3, T26; 4, B67; 5, T30; 6, B185; 7, B249; 8, B128 and 9, B7-1.

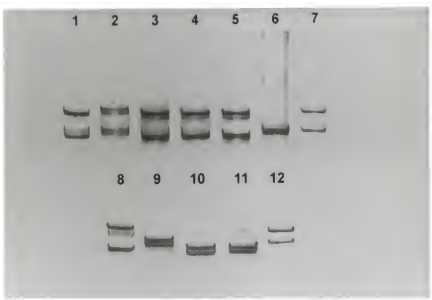


Figure 15. SSCP analysis of the p27 gene from other CTV strains. 1, T30; 2, B5; 3, B32; 4, B192; 5, B274; 6, B272; 7, T36; 8, B11; 9, B37; 10, B28; 11, B148 and 12, T36.

could be separated into nine different groups: 1) mild strains T30, T26, B67, B5, B32, B192 and B274, 2) mild strain B272, 3) severe, stem pitting B7 and B128, 4) severe, stem pitting strains B249 and B185, 5) severe, stem pitting strain B227, 6) severe, quick decline strain T36, 7) severe, quick decline strain B11, 8) severe, quick decline strain B37, and 9) severe strains B28 (stem pitting) and B148 (quick decline).

Discussion

The p27 genes of the CTV strains sequenced showed 86% or greater homology at the RNA level and 93% or greater at the amino acid level (deduced sequence). These values are similar to those found for the CP (Pappu et al., 1993b). Several of the nucleotide substitutions are consistently conserved only within a particular biological group (mild, severe or severe causing stem pitting). These distinct sequences may have potential for the development of group-specific molecular probes. Some of the nucleotide substitutions translated as nonsynonymous, although most of them were with functionally conserved amino acids.

The conservation of four amino acid substitutions between the severe strains compared to the mild suggests a possible relationship of p27 with symptom development. However, it is necessary to note that a limited number of strains were sequenced, and this in no way demonstrates the role of p27, if any, in symptomatology. Further experiments are necessary to

establish the relationship between these amino acids and biological activity. For example, transformed plants expressing p27 from severe CTV strains could be used to determine symptom expression when those plants are infected with mild strains.

Phylogenetic analysis of the amino acid sequences of p27 also indicated a strong relationship between sequence and biological activity, with mild and severe strains forming two distinct groups. Stem pitting strains B7-1, B128 and B249 also formed a distinct domain within the severe strains. Interestingly, RNA analysis showed a different relationship, with B227 forming a separate group from all other strains. The decline strain T36 was more similar to the mild strains T26, T30, and B67 than to strains causing stem pitting. This is an indication that the severe strains constitute a polyphyletic group, and that the similarities in protein sequence (and possible symptom development) represent an example of evolutionary convergence.

Comparison of the phylogenetic trees obtained with CP sequences showed different relationships, indicating distinct evolutionary rates for the two genes. From these results, the amino acid sequence of the p27 gene is more useful in predicting the biological reaction of an strain than CP sequence (RNA or amino acid).

Alignment of p27 and CP deduced amino acid sequences revealed that the C-terminal portion of the two proteins is

the most conserved. Located also in this region are the two consensus amino acids which are conserved in the CPs of filamentous viruses (Pappu et al., 1994). This portion must then contain important active domains for both proteins.

SSCP analysis of the p27 gene of strains T26, T30, T36, B67, B7-1, B128, B185, B227 and B249 produced groupings similar to those obtained in phylogenetic analysis of the RNA sequences. The only exception is the separation of the stem pitting strains into two groups with different patterns: B7-1 and B128; B249 and B185. Interestingly, B249 is more similar in RNA sequence to B7-1 (99.3%) or B128 (99.6%) than it is to B185 (98.8%). This indicates that certain nucleotide changes have more influence in conformation than others. Sheffield et al. (1993) found that the position of the base change was more important than the type of nucleotide substitution for the detection of such a change. Strains B227 and T36, the type strain for CTV, showed unique SSCP patterns.

The results for all the strains examined confirmed the sequence analyses indicating that mild strains were very homogeneous in sequence, regardless of the geographical origin. Only one strain, B272, a very mild strain from Colombia, showed a different SSCP pattern from all other mild strains. Severe, quick decline strains, on the other hand, were quite variable and separated into four different groups. Strains T36, B11 and B37 formed distinct SSCP patterns and B148 grouped with the stem pitting strain B28.

SSCP can detect single nucleotide changes in short DNA sequences (150 bp) (Orita et al., 1989; Sheffield et al., 1993), but is less efficient when longer fragments are analyzed. PCR amplification can be followed by restriction digestion to generate short fragments if longer sequences are amplified (Iwahana et al., 1992). For the CTV p27 gene this technique seems to be useful in differentiating certain strains. Furthermore, some of the patterns were highly conserved within particular biological groups, like the mild strains. This technique can then be used as a rapid and simple assay for characterization and identification of CTV strains. However, it is necessary to determine the variability in other regions of the genome to expand the possibilities for this characterization. The use of restriction enzymes, together with SSCP can be a useful way of analyzing longer portions of the genome, while reducing the costs in primer synthesis.

CHAPTER 4
TRANSFORMATION OF SWEET ORANGE WITH P27 AND P20

Introduction

Sequence analysis and the identification of ORFs in other plant viruses have permitted the use of some of those genes to transform plants and protect them against viral infections caused by similar strains. The first and most widely used gene has been the CP (Powell Abel et al., 1986; Beachy et al., 1990; Pappu et al., 1995). Transgenic plants expressing CP genes and showing various degrees of resistance have been obtained for members of at least a dozen different virus groups (Hull and Davies, 1992; Pappu et al., 1995), indicating that this is a relatively general phenomenon. Several non-structural viral genes also have been used to transform plants with mixed results in the induction of resistance. For example, three nonstructural genes of tobacco rattle tobravirus transformed into tobacco plants did not induce resistance to the virus (Angennent et al., 1990). On the other hand, tobacco plants transformed with a 54 kDa putative replicase protein of tobacco mosaic tobamovirus (TMV) were resistant to high concentrations of intact TMV and its RNA (Golemboski et al., 1990).

The finding of new and varied sources of resistance in food crops is important. Historically, pathogens frequently overcome the resistance incorporated into those crops, usually obtained by long selection processes. The incorporation of viral genes can also offer a source of resistance when those genes are not naturally present in the crop. In addition, the expression of viral genes in plants, even if those genes are not protective, is an important tool in determining the function of those genes. For example, the 30 kDa protein of TMV was shown to facilitate the cell-to-cell movement of a mutant strain of TMV (capable of replication but unable to cause systemic symptoms) when expressed in transgenic tobacco plants (Deom et al., 1987).

Most plant transformation experiments, with viral genes or others, have been accomplished in herbaceous, annual plants and only a few woody species have been successfully transformed. This is due to the technical difficulties in regenerating those plants, and the length of screening and testing for resistance. Plum (*Prunus domestica* L.) was recently transformed with the CP gene of plum pox potyvirus using *Agrobacterium* (Machado et al., 1994; Scorza et al., 1994). Papaya (*Carica papaya* L.), a perennial plant, has also been transformed with the CP gene of papaya ringspot potyvirus (Tennant et al., 1994). Transgenic plants, tested in the field for two years, showed no virus replication or movement when challenged with the same strain of virus used for

transformation. However, when the transgenic plants were inoculated with different viral strains they showed only a delay in symptom development (Tennant et al., 1994), a phenomenon observed previously in other CP transgenic plants (Beachy et al., 1990). For annual crops, a delay in symptom development can be sufficient to reduce losses caused by a virus, but for perennial crops this will probably be of little value since they are expected to be in the field for many years.

Several citrus species have reportedly been transformed by different means, including direct uptake of DNA by protoplasts (Schell, 1991), co-cultivation of cell suspensions with *Agrobacterium* (Hidaka et al., 1990), and infection of epicotyl segments with *Agrobacterium* (Moore et al., 1989; Kaneyoshi et al., 1994; Peña et al., 1995). There are two reports of citrus transformation using the CP gene of CTV (Gutiérrez et al., 1992; Schell et al., 1994). The plants obtained in these experiments are being tested currently for resistance to CTV (G.A Moore and J.W. Grosser, personal communication).

To study the feasibility of using some of the non-CP genes of CTV in *Agrobacterium*-mediated plant transformation, p27 and p20 genes were used to transform "Pineapple" sweet orange plants. Transgenic plantlets were selected after target gene expression was detected in infected citrus tissue.

Materials and Methods

Materials

Calf intestinal alkaline phosphatase, Photogene Nucleic Acid Detection System Version 2.0 and biotin-14-dATP were from GIBCO BRL (Gaithersburg, MD). Murashigue and Skoog tissue culture basal salt mixture (MS) and antibiotics were from Sigma Chemical Company (St Louis, MO). Mefoxin was from Merck Sharp & Dohme (West Point, PA). Plasmids pMON10098, pMON755 and *Agrobacterium* ABI were obtained from Monsanto (St Louis, MO). "Pineapple" sweet orange seeds were from Willits & Newcomb Inc. (Arvin, CA). The 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt (X-gluc) was supplied by Biosynth, Biochemica & Synthetica (Staad, Switzerland) or by Gold Biotechnology, Inc. (St. Louis, MO).

Cloning of p27 Into the Transformation Vector

CTV gene p27 was cloned into pMON10098 from the pUC118/p27 recombinant plasmid (Chapter 2). The gene contains the consensus sequence for optimal context of the AUG start codon (Kozak, 1986; Gallie, 1993). Approximately 0.5 μg of pMON10098 plasmid DNA and 2 μg of pUC118/p27 were digested separately with EcoRI (10 u) and XbaI (5 u) in 90 mM Tris-HCl pH 7.5 (at 37°C), 10 mM $MgCl_2$, 50 mM NaCl at 37°C for 4 h. Samples were purified in LMP agarose and ligated as explained in Materials and Methods, Chapter 2, except that the T4 polynucleotide kinase step was omitted. The plasmid was

transformed into *E. coli* DH5α competent cells and plated on 2 x YT agar plates containing 50μg/ml of spectinomycin.

Plasmid DNA increased and purified from the transformed colonies was screened for presence of the insert by digestion with EcoRI and electrophoresed in a 0.6% agarose gel with lambda/HindIII molecular weight markers. They were also screened by digestion with EcoRI and XbaI and electrophoresed in 0.8 agarose gels.

The gene for β-glucuronidase (GUS) was cloned into pMON10098/p27 from pMON755. Both plasmids (1 μg of DNA each) were separately digested with NotI (2.5 u) in 6 mM Tris-HCl pH 7.9 (at 37°C), 6 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT and purified in LMP agarose. Linearized pMON10098/p27 was treated with calf intestinal alkaline phosphatase (1.46 u) in 50 mM Tris-HCl pH 8.5, 0.1 mM EDTA according to the manufacturer's instructions and then extracted with phenol, phenol: chloroform, chloroform and precipitated with ethanol (Sambrook et al., 1989). Ligation of the GUS gene into pMON10098/p27 and transformation into *E. coli* DH5α was as described above.

To confirm presence and orientation of the p27 gene, the insert was sequenced following the protocol explained in Materials and Methods, Chapter 2. This plasmid was designated as pVF1 (Fig. 16).

Cloning of p20 Into the Transformation Vector

The cloning procedure for p20 was similar to that described above, but the GUS gene was cloned in pMON10098 first. The p20

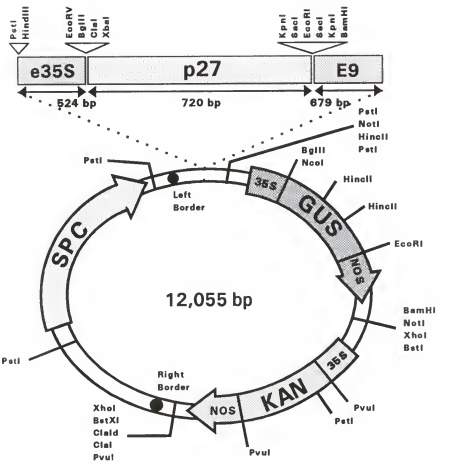


Figure 16. Restriction map of the pVF1 plasmid, containing p27.

gene was cloned subsequently from pUC118/p20 using XbaI and SacI restriction sites. The new plasmid was designated as pVF2 (Fig. 17).

Transformation of pVF plasmids into *Agrobacterium*

Transformation of *Agrobacterium* with the pVF plasmids was by using the triple mating procedure. Overnight, 3 ml cultures in LB liquid medium of the following bacteria were used: *Agrobacterium* ABI (supplemented with 25 μg/ml chloramphenicol, 50 μg/ml kanamycin), *E. coli* PRK 2013 (50 μg/ml kanamycin) and *E. coli* DH5α with either pVF1 or pVF2 (75 μg/ml spectinomycin). Samples of 250 μl from each culture were mixed in a microcentrifuge tube and centrifuged for 30 sec at 14,000 rpm. The pellets were resuspended in 50 μl of 10 mM $MgSO_4$ and placed on single LB agar plates free of antibiotics and without spreading the bacteria. The plates were incubated at 28°C for 24 h. Bacteria were collected from single plates and resuspended in 1 ml of 10 mM $MgSO_4$. Samples of 1, 5 and 10 μl were plated on LB agar containing 25 μg/ml chloramphenicol, 50 μg/ml kanamycin and 75 μg/ml spectinomycin and incubated for 3 to 4 days at 28°C. Surviving bacterial cultures were then kept on YEP (1% yeast extract, 1% peptone, 0.5% NaCl pH 7.0) agar plates containing the three antibiotics.

Screening of Transformed Bacteria

Agrobacterium colonies obtained in the transformation event were screened using PCR to determine the presence of the pVF plasmids. Samples from colonies were resuspended in 50 μl

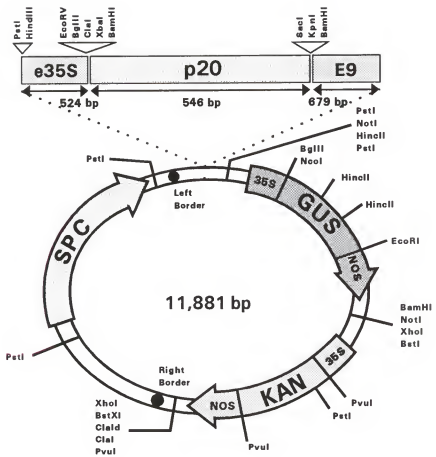


Figure 17. Restriction map of the pVF2 plasmid, containing p20.

of 20 mM Tris-HCl pH 8.5, 2 mM EDTA, 1% Triton X-100, boiled 75for 15 min and centrifuged for a few seconds. PCR reactions contained the normal constituents plus 1 μl of the bacterial extracts and the appropriate primers for p27, p20 or the GUS gene.

Transformation, Selection and Regeneration of Citrus Plants

Seed germination. The procedure for seed germination was modified from Moore et al. (1992, 1993). Intact seeds were surface sterilized (10 min in 70% ethanol, 20 min in 20% Clorox and 2 drops of Tween-20, rinsed 3 times with sterile distilled water) and germinated individually in 150 x 25 mm capped tubes containing 10 ml of half-strength MS basal salt medium, 2.5% sucrose, 0.005% myo-inositol, 0.8% agar pH 5.7 and maintained at 27°C with 16 h fluorescent light. Seedlings were used 3 to 6 months after sowing.

Bacterial culture. Individual colonies of *Agrobacterium* with either pVF1 or pVF2 were increased in 50 ml flasks containing 5 ml YEP medium with the three antibiotics and incubated overnight at 28°C and 200 rpm to post log phase. The bacteria were collected by centrifugation at 3,500 g for 15 min at 4°C and resuspended in 1.5 ml of YEP without antibiotics. The solution was kept on ice until its use for transformation.

Plant transformation. Nodal, internodal and root segments from "Pineapple" sweet orange seedlings were used as explants for transformation (Moore et al., 1992, 1993). The internodal

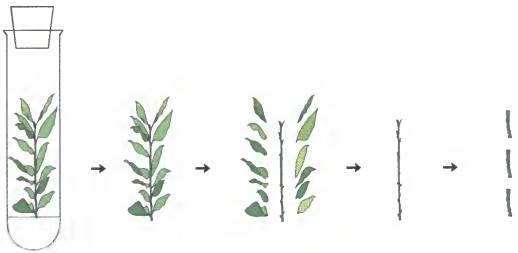
and root pieces (1 cm) were inserted on MS medium (MS basal salt, 5% sucrose, 0.01% myo-inositol, 0.8% agar pH 7.5) with the apical end protruding. Nodal segments were placed lengthways on the MS medium. The explants were inoculated with one drop of *Agrobacterium* using a syringe and co-cultivated for 3 days at 27°C and 16 h of fluorescent light (Fig. 18, A and B).

Selection. After 3 days, the explants were transferred to petri plates (20 x 100) with MS medium, without growth regulators, and supplemented with 100 μg/ml of kanamycin and 200 μg/ml of mefoxin. After 4 to 6 weeks, shoots began to emerge from the segments. Shoots were harvested and explants transferred to fresh selection medium every 4 weeks for up to three months, when they were discarded (Fig. 18, C).

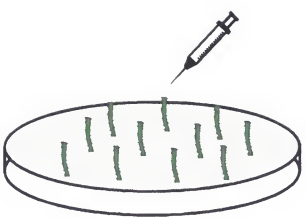
Analysis of shoots. Harvested shoots were assayed histochemically for GUS activity (Stomp., 1992). Segments excised from the basal end of the shoots were incubated, using microtiter plates, in 25 μl of 0.1 M $NaPO_4$ buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.25 mg/ml X-gluc at 37°C overnight. Transformed tobacco leaf segments, containing the GUS gene (kindly provided by G. Moore), were included as positive controls. The tissue samples were fixed and destained with 50 μl of 95% ethanol: acetic acid (3:1) at room temperature for approximately 1 h before examination under the microscope.

PCR analysis. Some of the GUS positive (GUS+) tissue samples were further analyzed by PCR using p27 or p20 specific

Figure 18. Schematic representation of the transformation protocol for sweet orange plants. A), seedlings (3 to 6 months old), germinated in culture tubes, were pruned to eliminate leaves. Segments of approximately 1 cm were cut out from roots and stems (separating nodal and internodal portions). B), explants were placed in culture plates containing MS medium with the apical end protruding (internodes and roots) or length-ways on the medium (nodes). Explants were then inoculated with one drop of *Agrobacterium*. After three days of incubation the explants were transferred to selection medium (MS with antibiotics). C), approximately four to six weeks after the transformation shoots started to regenerate. The shoots were excised from the explants and assayed for GUS activity.



A



B

C

primers (Chapter 2). Fixed materials were washed 4 times (30 min each) with 200 μl of sterile distilled water and DNA extracted using the procedure of Rogers and Bendich (1988). The total, unquantified DNA extracted from each sample was used in a 100 μl PCR reaction containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 10 mM DTT, 0.1 mM dNTPs, 2.5 u Taq polymerase and 40 pM of each primer. The mixtures were given 25 cycles of incubation at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final incubation cycle at 94°C for 1 min, 50°C for 1 min and 72°C for 10 min. Approximately 30 μl of the PCR product were analyzed by 0.8% agarose gel electrophoresis.

Southern analysis of the PCR products (1 μl) was conducted using the procedure described in Sambrook et al. (1989). The membranes were probed using the Photogene system. Biotinylated p27 probes were produced using pVF1 clones as templates in PCR reactions (Rashtchian and Mackey, 1992; Mertz et al., 1994).

Regeneration of shoots. GUS positive shoots were transferred to sterile baby food jars containing 40 ml of sterile soil (Metro-mix 200) and irrigated with 15 ml of sterile two-thirds strength MS basal salt medium. A solution of 100 μg/ml kanamycin, 200 μg/ml mefoxin and RooTone (Green Light Co., San Antonio, Texas) was applied to the base of the shoots before planting into soil. Plants were maintained at 27°C with 16 h fluorescent light.

Results

Cloning of CTV Genes Into the Transformation Vectors

Several approaches were used to determine the presence and copy number of the CTV genes in the pVF plasmids. Restriction digestion of pVF1 and pVF2 with EcoRI and XbaI released inserts of the expected sizes indicating the presence of the gene. Comparison of the plasmid sizes after linearization with EcoRI indicated the presence of only one copy in each plasmid. Finally, sequence analysis also confirmed the presence and orientation of the CTV genes in the pVF plasmids (data not shown).

PCR with p27, p20 (Fig. 19) or GUS specific primers indicated the presence of the pVF plasmids in *Agrobacterium*. This assay was employed routinely to determine the presence of the insert of interest in the cultures to be used in transformation experiments.

Transformation and Regeneration of Sweet Orange

Three different types of explants were used for transformation. Nodes and internodes were used with both pVF1 and pVF2, and roots were only used with pVF1. This may be the first report of the use of root segments for transformation and regeneration of sweet orange. They were included for two reasons, to increase the number of explants obtainable per seedling and to determine their regenerative capacity. Figure 20 shows adventitious shoots on internodal segments and GUS+ shoots on soil for rooting.

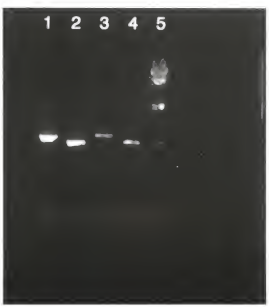


Figure 19. PCR of *Agrobacterium* ABI cultures to confirm the presence of pVF plasmids. Lane 1, *Agrobacterium*/pVF1 bacterial extract amplified using p27 primers; Lane 2, *Agrobacterium*/pVF2 bacterial extract amplified using p20 primers; Lane 3, purified pVF1 plasmid DNA amplified with p27 primers; Lane 4, purified pVF2 plasmid DNA amplified with p20 primers; Lane 5, Lambda DNA digested with HindIII used as a MW marker.

A

B

Figure 20. Adventitious shoot formation on internodal segments (A) and GUS+ shoots in soil for rooting (B).

The results in Table 5 indicate that more adventitious shoots were produced from root segments compared to nodes or internodes. Nodes produced the lowest average number of adventitious shoots. There was no difference in shoot production when explants were inoculated with pVF1 or pVF2. However, comparison of the efficiencies of transformation (as percentage of GUS+ shoots obtained from the total) showed that internodes were more efficiently transformed with pVF1 (Table 6) than nodes or roots. This indicates that proportionately, internodes generated twice as many GUS+ (Fig. 21) shoots than the root segments. Interestingly, transformation with pVF2 was more successful on nodes than on internodes (Table 6). Again, taking regeneration and transformation efficiency together, internode segments produced three times as many GUS+ shoots than nodes.

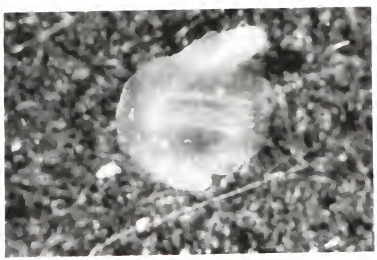
Transformation efficiency was quite variable between experiments, ranging from 0 to 15%. The overall efficiency was lower than desirable. Taking the results from all the transformation experiments using pVF1, from 2,481 shoots obtained, 148 (5.97%) were GUS+. When pVF2 was used from 1181 shoots obtained, 71 (6.0%) were GUS+.

Survival of the GUS+ shoots in soil (Table 6) ranged from 25 to 45%, seven months after transfer, although none of them have rooted at the time this is written. The results from the shoots obtained from root segments are not included as they have been in soil only two months.

Table 5. Effects of explant type and plasmid on adventitious shoot formation in "Pineapple" sweet orange.

EXPLANT	PLASMID	No. OF SEGMENTS	SHOOTS PER SEGMENT
Nodes	pVF1	350	0.30 ± 0.11
	pVF2	378	0.34 ± 0.15
	Total	728	0.32 ± 0.14
Internodes	pVF1	910	1.14 ± 0.50
	pVF2	434	1.00 ± 0.38
	Total	1344	1.10 ± 0.47
Roots	pVF1	42	1.64 ± 0.45

A



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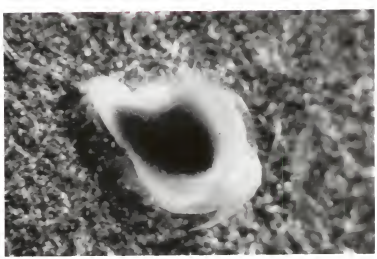

Figure 21. GUS assay on adventitious shoots of "Pineapple" sweet orange regenerated from *Agrobacterium*-inoculated internodal segments. A), GUS- segment. B), GUS+ segment.

Table 6. Transformation efficiencies and survival of shoots of "Pineapple" sweet orange using pVF1 (p27) and pVF2 (p20).

PLASMID[a]	EXPLANT	No. OF SHOOTS	No. OF GUS+ SHOOTS	% GUS+ SHOOTS	No. SURVIVING	% SURVIVING[b]
pVF1	Nodes	148	1	0.7	0	0.0
	Internodes	1037	71	6.8	18	25.4
	Roots	208	5	2.4	--[c]	--[c]
pVF2	Nodes	215	19	8.8	7	37.8
	Internodes	886	49	5.3	22	44.9

a pVF1=p27, pVF2=p20.
b Survival in soil seven months after transfer.
c Adventitious shoots derived from root segments have been in soil less than seven months.

To confirm presence of the CTV genes in the GUS+ plants, samples were analyzed using PCR (Fig. 22, Table 7). For those GUS+ shoots transformed with pVF1, 31% were also PCR+ . For pVF2, 37% of the GUS+ shoots were also PCR+. Transformed, GUS- segments were used as negative controls to rule out the possibility of obtaining PCR products from contaminating *Agrobacterium*. Hybridization of the PCR products obtained from the plants with p27 or p20 probes demonstrated they were CTV specific sequences (Fig. 23).

Discussion

The number of shoots produced by the internodes is comparable to what was obtained previously for Carrizo citrange [*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.] under similar experimental conditions (Moore et al., 1992). There are no published values for root and node segments. Comparing the three different types of explants used to transform with pVF1, root segments produced more adventitious shoots than internodes, and internodes more than nodes. However, a higher percentage of GUS+ shoots were obtained from internodes than from roots, making internode segments more efficient. For pVF2 internode segments also produced more GUS+ shoots than nodes.

Kanamycin was used in the media for selection of transformed shoots, however, only an average of 6% (considering all experiments together) were GUS+. "Escapes"

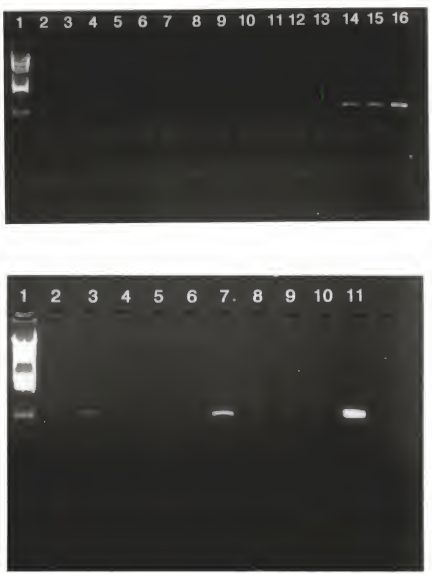

Figure 22. PCR amplification of DNA extracts from "Pineapple" sweet orange GUS+ segments transformed with p27 (A) or p20 (B). A), Lane 1, Lambda DNA digested with HindIII used as a MW marker; Lanes 2 and 7, GUS- segments; Lanes 3 to 6 and 8 to 15, GUS+ segments; Lane 16, pVF1. B), Lane 1, Lambda DNA digested with HindIII used as a MW marker; Lane 2, GUS- segment; Lanes 3 to 10, GUS+ segments; Lane 11, pVF2.

Table 7. Number and percentage of "Pineapple" sweet orange shoots that tested positive for GUS and by PCR.

PLASMID	GUS+	PCR+	% PCR+
pVF1	79	25	31.2
pVF2	8	3	37.5

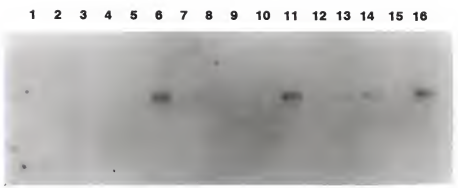

Figure 23. Southern analysis of PCR products from GUS+ segments of "Pineapple" sweet orange hybridized with a p27 specific probe. Lanes 1 and 12, GUS- segment; Lanes 2 to 11 and 13, 14, GUS+ segments transformed with p27 gene; Lane 15, Lambda DNA digested with HindIII; Lane 16, amplified p27 from pVF1.

have been reported previously (Moore et al., 1992; Peña et al., 1995) and constitute a serious problem, increasing the work necessary in screening the shoots. This ineffective Kanamycin selection may be explained by one or several possibilities: 1) protection of non-transformed cells by the surrounding transformed cells, 2) contamination with *Agrobacterium* or 3) by endogenous levels of NPT II activity (Jordan and McHughen, 1988; Dandekar et al., 1988; Moore et al., 1992; Peña et al., 1995).

Transformation efficiencies, expressed as percentage of GUS+ shoots from the total adventitious shoots produced, were very variable between experiments and in general low. Values ranged from 0 to 15%, with an average of 6%. These results are comparable to those obtained by Moore et al. (1992, 1993), but lower than the 55% obtained with Carrizo citrange by Peña et al. (1995) or the 55% to 87% reported by Kaneyoshi et al. (1994) with *Poncirus trifoliata* Raf. One of the differences in the transformation protocol used here and the ones mentioned above is that the segments are inoculated by submersion for several minutes to a few days in a solution containing the *Agrobacterium*. Another difference is that in those cases the explants were obtained from relatively young seedlings (3 to 5 weeks old) instead of 3 to 6 months old as reported here. Finally, the procedure of Kaneyoshi et al. (1994) includes acetosyringone in the inoculation media to increase the efficiency of bacterial infection.

Rooting of the transformed shoots has been difficult, with lower survival rates than reported by other authors (Moore et al., 1992; Kaneyoshi et al. 1994; Peña et al., 1995). This may be due to a detrimental effect of the antibiotics on the explants or possibly to the expression of the CTV gene products in the shoots. CTV infection reduces root and bud formation of shoots *in vitro* (Duran-Vila, 1989). It is possible that the constitutive expression p27 or p20 causes the reduction of root formation in the explants.

PCR analysis of the same segments used in the histochemical GUS assay confirmed the presence of p27 and p20 in the putatively transformed shoots. Only 31% (p27) or 37% (p20) of the GUS+ segments were also PCR+. This may be due to true false positives, or to deficiencies in the DNA extraction procedure because of the small amount of tissue used. Hybridization experiments confirmed the specificity of the bands obtained in the PCR analysis, demonstrating that they are p27 and p20 genes.

CHAPTER 5
SUMMARY AND CONCLUSIONS

1) The expression of CTV ORFs p27 and p20 was detected *in vivo* in CTV-infected plants using specific polyclonal antibodies raised against the recombinant proteins.

2) Cell fractionation analysis indicated that p27 accumulates in the cell wall fraction, whereas p20 accumulates in the soluble protein fraction.

3) Tissue blots indicated that p27 is present in the phloem. This is in agreement with CTV being a phloem limited virus.

4) The function of p27 is still unknown, but based on sequence homology and cell fraction studies, p27 could be involved in any or all of three functions: virus assembly, virus movement and aphid transmission.

5) Based on sequence conservation, the active site of p27 appears to be located in the C-terminal portion of the protein.

6) The function of p20 is also unknown. Its *in vivo* expression and accumulation did not give any indications about its possible role in the CTV life cycle.

7) Sequence and phylogenetic analysis of the p27 gene from different CTV strains indicated a relationship between deduced amino acid sequence and biological activity of the strains.

8) SSCP analysis permitted the differentiation of CTV strains. Most mild strains could to be identified using this method.

9) The genes encoding p27 and p20 were transformed into "Pineapple" sweet orange using *Agrobacterium*. Transformation was confirmed using GUS and PCR assays.

10) Internodal segments from seedling epicotyls were the most efficient in producing GUS+ adventitious shoots.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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